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Studies in Stomatal Behaviour

VI. AN INVESTIGATION OF THE LIGHT RESPONSES OF WHEAT STOMATA WITH THE ATTEMPTED ELIMINATION OF CONTROL BY THE MESOPHYLL

PART I. *Effects of Light Independent of Carbon Dioxide and their Transmission from One Part of the Leaf to Another*

O. V. S. HEATH AND J. RUSSELL

Research Institute of Plant Physiology, Imperial College of Science and Technology, London, S.W. 7

Received 1 March 1953

SUMMARY

An apparatus is described enabling light responses of wheat stomata to be followed by periodical porometer readings, between which the substomatal cavities can be swept with carbon dioxide-free air forced in through the stomata. This is held to eliminate the influence of changes in the carbon dioxide content of the intercellular space atmosphere brought about by photosynthesis and respiration in the mesophyll. Using this apparatus in an experiment of 'Latin square' design with 6-fold replication of three light treatments, a statistically significant response to light was found in a leaf area subjected to varying light intensities from 90 to 800 f.c. This response was reflected in a similar significant effect, though of smaller magnitude, occurring in a similarly swept 'control' area 2 cm. distant and maintained at a constant light intensity of 270 f.c., the intervening portion of leaf being illuminated with 800 f.c. throughout. It is argued that this transmission of a light effect from one area to another does not occur via the intercellular spaces, nor by means of carbon dioxide gradients from cell to cell. It is concluded from the data that there is an indirect effect of light and perhaps also a direct effect upon the guard cells themselves; it is probable that neither of the two effects concerned operates by reduction of the internal carbon dioxide content in the guard cells.

INTRODUCTION

EARLIER investigation of stomatal response to variation of carbon dioxide concentration of the external air was reviewed in this journal by Heath (1950), with the notable omission of the work of Freudenberger (1940), of which the author was unaware owing to the fact that the war-time volumes of Protoplasma were not available. She had carried out experiments with *Canna* and *Sanchezia* using a porometer (apparently that of Darwin and Pertz, 1911) with air of various known carbon dioxide contents swept through the cup between the readings, which were generally taken at intervals of about an hour. In general she found stomatal opening in response to decrease of carbon dioxide and closure with increase using concentrations of zero, 0·003, 0·015,

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0·03, and 2·5 per cent., but the magnitude of the movement bore no apparent relation to the magnitude of the change of concentration. The many inconsistencies in this respect were doubtless due to the fact that she did not wait for equilibrium to be established under one set of conditions before changing to another, the importance of which has been stressed elsewhere (Heath, 1950, p. 60), and her results are thus of a qualitative nature only. Unlike Heath and Milthorpe (1950, see below) she did not record a maximal opening at any concentration above zero carbon dioxide, possibly because, out of the three experiments presented employing 0·003 per cent. carbon dioxide, two were carried out in darkness. In the next paper of this series it will be shown that in the dark zero carbon dioxide gives much greater opening than 0·01 per cent.

Heath (1948, 1950) found that reduction of carbon dioxide content in the external air below the normal caused marked stomatal opening in *Pelargonium* and wheat. For *Pelargonium* evidence was obtained that the carbon dioxide content operated from the sub-stomatal cavity and not via the outer walls of the guard cells, for stomata completely closed in darkness did not show the usual opening response to carbon dioxide-free air. This observation has been confirmed by Scarth and Shaw (1951) for the same species. A preliminary quantitative investigation with wheat by Heath and Milthorpe (1950) showed at each of three light intensities (975, 625, and 275 f.c.) marked stomatal opening from 0·03 to 0·01 per cent. external carbon dioxide but no further appreciable response between 0·01 per cent. and zero concentration. They interpreted this as meaning that an *intercellular space* carbon dioxide content of 0·01 per cent. gave maximal opening and drew attention to the assimilation experiments of Heath (1939, 1949, 1951) and of Gabrielsen (1948) in which a carbon-dioxide concentration of about 0·01 per cent. was indicated as the minimum that could occur in the intercellular spaces of leaves during photosynthesis. These responses to external carbon dioxide concentration thus illustrate the mode of operation of changes of light intensity in controlling stomatal movement *indirectly* by means of the intercellular space atmosphere as affected by photosynthesis and respiration in the mesophyll cells. They also found, however, evidence for an important effect of light on stomatal movement independent of the intercellular space carbon dioxide. Although this light effect might operate from the epidermal or even the mesophyll cells, the simplest hypothesis would seem to be that it is a *direct* effect acting upon the guard cells themselves.

Scarth and Shaw (1951) present results for a *Pelargonium* leaf exposed to 0·03 per cent., 0·026 per cent., and zero carbon dioxide concentration at 400 f.c. light intensity and to 0·023 per cent. and 0·015 per cent.[1] in addition at 50 f.c. Carbon dioxide-free air gave maximal stomatal opening at each light intensity, but as noted 0·01 per cent. carbon dioxide was not tried. Their paper is mainly concerned with the relation between stomatal movement and photosynthesis, and they conclude that their results are 'consistent with the view that the main action of light on stomata operates through photosynthetic reduction of CO_2 concentration within the leaf'.

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[1] Assuming that their 'normal' air contained 0·03 per cent.

In the subsequent paper (Scarth and Shaw, 1951*a*), however, they refer to 'the question of whether the direct action of light on stomata, as distinguished from its indirect action through photosynthesis in the mesophyll is also photosynthetic', and they describe an experiment in which slow starch formation was recorded for the dark-starved guard cells on white portions of variegated *Pelargonium* leaves when exposed to light with the green portions covered. Alvim (1949) also had stated that stomata of *Zebrina*, *Phaseolus*, and *Nicotiana* on detached strips of epidermis in water showed an increased starch content after some days in light in the presence of carbon dioxide, but in its absence all starch disappeared. It thus appears that guard cells are capable at least of slow photosynthesis. The present authors are, however, unaware of any experimental evidence so far published that photosynthesis in the guard cells plays a part in the normal light response of stomata, although this was the mechanism originally postulated by von Mohl (1856) and has been much discussed in the absence of evidence since that date. As for direct effects of light upon the guard cells independent of chlorophyll, the results of Wilson (1948) for light response in etiolated *Coleus* leaves with apparently chlorophyll-free guard cells and mesophyll as tested with the fluorescence microscope would suggest that these can occur.

These results of Wilson's and those of Heath and Milthorpe mentioned above would seem to be the only evidence presented so far for a direct effect (either photosynthetic or non-photosynthetic) of light upon the guard cells in causing the normal light response of stomata. As noted elsewhere (Heath, 1949, pp. 190 and 205), stomata on detached strips of epidermis have not as yet been found to respond normally to light,[1] though this is not perhaps solely due to the absence of the photosynthetic activity of the mesophyll. Responses on the white parts of variegated leaves may always be due to the influence of the chlorenchyma if the green parts are similarly illuminated; thus Scarth (1932) working with variegated leaves of *Pelargonium*, *Abutilon*, *Pandanus*, and a species of grass found that '. . . little effect may be produced in the white parts. The stomata remain closed or open slightly, except that *near the boundary of the green, the stomata on the white areas open well and the influence spreads, as exposure to light continues*' (italics in original). He concludes: '. . . it is clear from these experiments that stomata separated from assimilating tissue are only slightly affected by the action of visible light of any wave length'. A further criticism of many experiments with variegated leaves arises from the doubt as to whether the white portions of the lamina are really free of chlorophyll. In spite of these criticisms, one of them due to Scarth himself, in support of the hypothesis that assimilation in the guard cells causes a light response, Scarth and Shaw (1951*a*) present data from an experiment in which the time curves of stomatal pore width (by Lloyd's, 1908, method) for the white and green areas of a variegated *Pelargonium* leaf during 24 hours under outdoor conditions showed a coincident 9 a.m. peak and midday trough. Other peaks and troughs did not coincide, however, and in any case estimations of stomatal width were made only at hourly intervals, whereas the diffusion of carbon dioxide between white and green areas should be a matter of minutes or even seconds. It cannot therefore be admitted that 'the synchronisation of the response in the two areas supports other evidence that opening in the white area is dependent on photosynthesis in the guard cells'. The 'other evidence' referred to is all of the type that conditions which suppress photosynthesis in green leaves (e.g. chloroform vapour) cause stomatal closure, but all such data could *equally well* support stomatal control by the intercellular space atmosphere. In the next paper of this series some evidence will be presented of which the simplest interpretation is that photosynthetic reduction of carbon dioxide in the guard cells does play a part in the normal light response of wheat stomata, and the authors believe that this is probably true. No good purpose, however, is served by the unqualified admission in support of the hypothesis of evidence which is so obviously ambiguous as to be no evidence at all; the uncritical acceptance of such evidence is likely in time to hinder further investigation by leading to false assumptions.

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[1] The response found by Williams and Shipton (1950) for *Pelargonium* stomata on detached epidermis in salt solutions does not, as the authors point out, appear to be a normal light response.

The considerable amount of information yielded by the preliminary and only partially replicated factorial experiments of Heath and Milthorpe (1950) suggested that a more comprehensive factorial design, adequately replicated, might well repay the large amount of labour involved. In Heath and Milthorpe's experiments, as in those of Freudenberger and of Scarth and Shaw, only the initial carbon dioxide contents in the air supplied to the leaf were controlled and the actual concentrations across the leaf surface, and still more those at the inner surfaces of the guard cells, must in most cases have differed appreciably from these owing to photosynthesis and respiration. They therefore suggested that for further investigation of these problems the carbon dioxide concentration in the substomatal cavities should be controlled to known values by passing air actually *through* the stomata, somewhat as in the 'through' assimilation experiments of Heath (1939). The present paper describes an attempt to carry out this suggestion using carbon dioxide-free air at three light intensities in order to obtain further evidence for or against the existence of a direct light effect upon the stomata; in the next paper a more complex experiment along similar lines but involving the interaction of light intensity with carbon dioxide will be described.

MATERIALS AND METHODS

1. *Plant material.* All experiments were carried out on potted plants of the same variety of wheat ('Charter') as was used in most of the experiments of Heath and Milthorpe (1950). Seeds were sown, eight to a 6-in. pot, in John

Innes potting compost No. 1 with a little sand mixed in the upper layers, and the plants were thinned to five per pot a week after germination.

The plants were grown in a greenhouse; in winter this was heated to 15° C. and a 1000-W. tungsten-filament lamp with reflector was then used to increase the light intensity to about 200–600 f.c. at the leaf surface and extend the day length to 14·5 hours. Experimental work was performed before elongation of the axis, on the flag leaf or on one of the two immediately preceding leaves; during the summer months plants were ready for use in 4–6 weeks and during the winter in 6–8 weeks from the time of sowing.

2. *Controlled conditions cabinet.* All the experiments were carried out in a plant chamber $51 \times 51 \times 76$ cm. high, equipped for the control of temperature, light intensity, and humidity. The temperature was controlled at $25{\cdot}5 \pm 0{\cdot}5^\circ$ C. by means of a Sunvic Type T.S. 3 thermostat and Sunvic Type F. 102–4 Relay; heating was effected by resistance mats between the inner and outer walls of the chamber. Illumination was supplied by an Osram 500-W. Horizon Type projector lamp, in an adjustable holder and surmounted by a parabolic mirror, enclosed in a separate compartment above the actual plant chamber. The lamp was air-cooled by means of a Ventaxia ventilating fan, and radiant heat was absorbed by a perspex water screen 12 mm. deep covering the whole top of the plant chamber. Distilled water was circulated through the screen in a closed system, including a small rotary pump and a coil of lead compo tubing immersed in a tank of constantly renewed tap-water above the chambers. The use of a closed system enabled the water in the cooling screen to be kept clean and free from algae. The plant chamber was aerated by an air-flow of 32 l./hour supplied from outside the laboratory building by means of an electrically driven diaphragm air pump (Austen 'Dymax'). Heat from the pump was removed from the air-flow by passing it through a coil of compo lead tubing in the cold water tank. Inside the plant chamber the air was passed through a spiral bubbler containing saturated calcium nitrate solution to maintain the relative humidity at about 50 per cent. Owing to the slow rate of air movement the humidity at the leaf surfaces not enclosed in the leaf chambers must have been considerably higher than this.

3. *Porometer.* The Wheatstone Bridge porometer (Heath and Russell, 1951), which was used throughout these experiments, was built on to the outside of the plant chamber. The three resistances R_F (loc. cit., Fig. 1) were situated inside the plant chamber and were thus maintained at approximately the same temperature as the leaf (R_L). The other fixed resistance (R_f) and the needle valve (R_v) together with the differential manometer were outside the chamber and thus at laboratory temperature. The porometer was calibrated in Gregory and Pearse units.[1]

4. *Leaf chambers.* In these experiments the aim was to maintain as far as possible known carbon dioxide contents (zero in the present case or different

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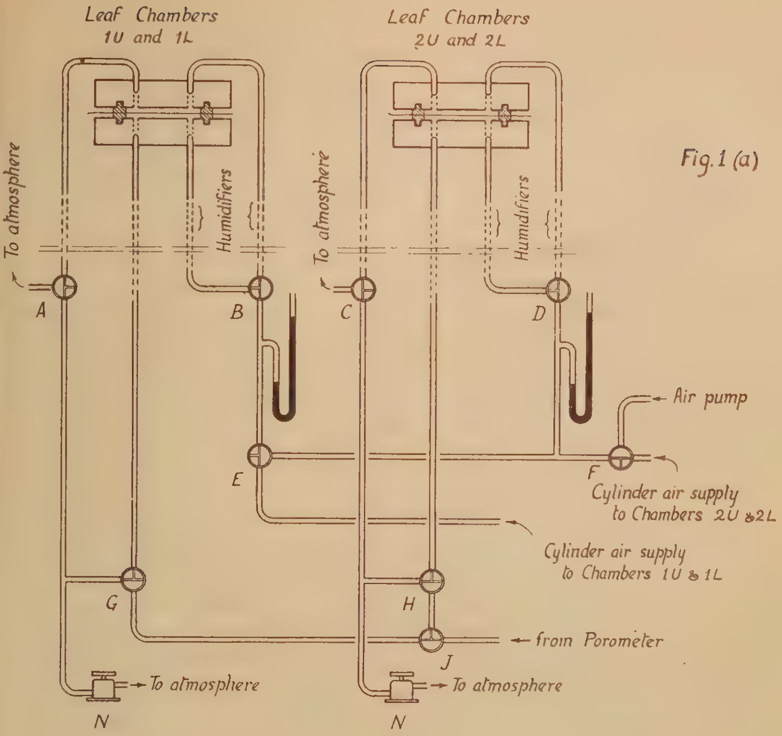
[1] Unit resistance equals that of a cylindrical tube for which $l/r^4 = 3{\cdot}77 \times 10^8$ cm.$^{-3}$ (Heath, 1939).

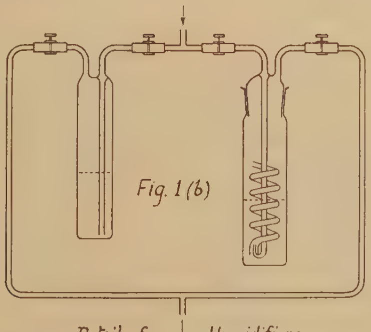
values in the subsequent experiments) in the substomatal cavities of those stomata which were mainly concerned in the porometer determinations. In wheat the ratio (α) of the stomatal number on the upper surface to that on the lower is actually greater than unity, being about 1·3 in the variety used. As pointed out elsewhere (Heath and Milthorpe, 1950) this results in nearly the whole of the air-flow due to the use of a porometer passing straight through from one surface to the other above the porometer cup and relatively little laterally through the leaf to (or from) the area outside the cup. The essential was thus to condition the stomata within and immediately above the cup, those outside the cup area being relatively unimportant. The principle used was therefore to attach a pair of identical transparent chambers above and below the leaf, force air of known carbon dioxide content into the leaf from both upper and lower chambers with an equally increased pressure, and allow it to escape laterally through the intercellular space system and thence through the stomata outside the cup area. At suitable intervals the flow could be interrupted and the lower chamber used as a porometer cup.

Pairs of chambers of the type described in detail by Heath and Milthorpe (1950), but enclosing an area of $1{\cdot}85 \times 0{\cdot}6$ cm.^{2}, were used (Fig. 1). Two such pairs of chambers formed at the two ends of a single pair of perspex blocks and 2·0 cm. apart were used on the same leaf. By the use of neutral filters (p. 8) one pair was maintained at a constant light intensity as a 'control' while the other was subjected to the different intensities constituting the treatments. Allowing for the overlap at the edges of the chambers, the intervening length of unenclosed leaf subjected to the full light intensity at all times was 1·6 to 1·8 cm.

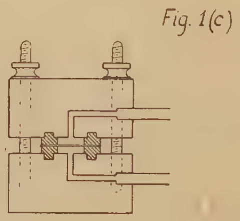
5. *Air supplies to the leaf.* Various carbon dioxide contents and rates of air-flow were made available by a method similar in principle to that of Heath and Milthorpe (1950) and Gregory *et al.* (1950). A cylinder of compressed air of previously determined carbon dioxide content supplied air through a fine adjustment valve to a 10-litre jar. Here the pressure was maintained constant by an escape valve consisting of a T-piece dipping in mercury to a depth of 12 cm., the jar acting as a reservoir and damping out minor fluctuations of pressure due to air bubbling through the mercury. Leaving the jar the air-stream was divided into two, one part passing through a bead tower containing normal caustic soda solution (renewed for each 1 or 2 days' experimentation) and the other through a bead tower containing distilled water. By recombining these two streams in various proportions, using flow meters and needle valves, any desired concentration of carbon dioxide between the cylinder concentration and zero, at any desired total rate of flow, could be obtained.

The rate at which air can be forced through the leaf in the manner described above depends upon the pressure, area, stomatal apertures, and numbers, and also on the width of the washers surrounding the chambers which define the minimum lateral path inside the leaf. Using gelatine washers for the side walls of the chambers it was found that a pressure of 10 cm. of





Detail of Humidifiers



Detailed side view of Leaf Chambers.

FIG. 1 (*a*). Diagram showing method of control of air-supplies to the leaf chambers. The portion of the diagram above the broken horizontal lines refers to the interior of the plant chamber. For further explanation see text. (*b*) Detail of humidifiers. The spiral bubbler contains saturated salt solution and the other bubbler distilled water. (*c*) Transverse section through a pair of leaf chambers.

mercury on both sides of the leaf could be maintained, but 5 cm. proved more practicable and this pressure would give a total flow of at least 250 c.c. per hour with most of the stomatal resistances encountered.

The arrangements for controlling the air supplies to the two pairs of leaf chambers (1*U*, 1*L* and 2*U*, 2*L*) were essentially as shown in Fig. 1*a*, but the two needle valves *N*, *N* were dispensed with for the present experiment. Between porometer readings the exit tubes to the chambers were shut off by taps *A*, *G*, *C*, and *H* and a flow through the leaf in each pair of chambers of 250 c.c./hr. of CO_2-free air was maintained constant by varying the pressures with changing stomatal resistances.

6. *Humidity and equilibrium values of stomatal resistance.* During an experiment, porometer readings were taken every 15 minutes and these were continued under each experimental treatment until the stomata had reached an equilibrium position before proceeding to the next. An equilibrium value, as used in the analysis of the results, was defined as the mean of three successive readings, in terms of $\log_{10}$ (resistance), varying by not more than 0·02; i.e. at 'equilibrium' there was a relative change of resistance of not more than 5 per cent. in half an hour.

The design of the experiment made it essential to obtain equilibrium readings for three successive treatments in each day's experimentation and preliminary experiments were carried out to find a suitable humidity for the 'through' airflow. With 100 per cent. R.H. the stomata exhibited a continuous drift without coming to equilibrium positions. Using 90 per cent. R.H., obtained by passing the air-streams through spiral bubblers containing saturated zinc sulphate solution, some 4 to 5 hours were required for each equilibrium value; 50 per cent. R.H. obtained with saturated calcium nitrate solution reduced this time to some 2 to 3 hours, and this was used in the present experiment.

The adjustment of humidity was effected inside the cabinet where the air-flow would have reached approximately 25° C. The volume of liquid in the spiral bubblers was balanced to give an approximately equal flow to the upper and lower chambers in each case. Volume changes in the saturated solutions were slight and not sufficient to cause appreciable difference of resistance to air-flow in the course of an experiment.

7. *Illumination in the leaf chambers.* Using a photo-electric light meter the position of the potted plants in the plant chamber was adjusted so that each upper leaf chamber received 1,000 f.c. light intensity in the focus of the parabolic mirror; this was made possible by the long straight filament of the 500-W. Horizon Type Projector lamp. The other leaves were at a lower light intensity, of the order of 500 f.c. To reduce the intensities in the control and experimental areas for the different light treatments, filters of ground glass and tissue-paper or filter-paper were used; when placed in a beam of 1,000 f.c. the former reduced the intensity to 330 f.c. and the latter to 110 f.c. Subsequent determination of the intensities actually within the leaf chambers showed that these were 800, 270, and 90 f.c. respectively. To change the light

intensities during an experiment by means of the filters the plant chamber had to be opened, but the temperature drop was made up in about 5 minutes and no effect was observed on the porometer readings.

8. *Experimental procedure.* The experimental leaf was set up in the leaf chambers the previous night; each chamber was connected to the porometer in turn and the nuts were slowly tightened until a constant reading was obtained, air-tight joints thus being produced without risk of crushing the leaf.

The position of the potted plant in the plant chamber was then adjusted for illumination and the leaf chambers connected up as in Fig. 1. At night the enclosed leaf areas were supplied with a saturated 'over' air-flow of 8 l./hour to each of the four chambers. This was done by turning taps *F* and *E* 90° clockwise from the position shown in Fig. 1, connecting all chambers to the pump drawing in outside air. All four spiral bubblers of saturated salt solution were clipped off and the air passed through distilled water in the alternative bubblers. The lines containing the taps *A*, *G*, *C*, and *H* were disconnected so that the air-stream after passing through the leaf chambers could aerate the plant chamber as a whole.

In the morning, at 9.0 a.m., taps *F* and *E* were returned to the position shown in Fig. 1 and the tubes reconnected. The pump then aerated the plant chamber directly, through a T-piece, as described on p. 5. Initial porometer readings were taken and the 250 c.c./hour 'through' flow of carbon-dioxide-free air begun; this was humidified to 50 per cent. R.H. by passage through the four spiral bubblers containing saturated calcium nitrate solution.

To take porometer readings, the air supply was first cut off by turning taps *B* and *D* to a 45° position; the pressure then built up to 12 cm. of mercury in the tubes behind the taps and excess air escaped via the T-piece dipping in mercury (p. 6). Taps *A* and *C* were then turned to open the upper leaf chambers to the atmosphere, and taps *G* and *H* to give unbroken leads to the lower leaf chambers. Using tap *J* the porometer could then be connected to either of the lower chambers as required. The interruption of the air-flow for the two porometer readings varied from 1 to 3 minutes. After porometer readings, before opening taps *B* and *D* to resume the air-flow, all other taps were returned to their previous positions. If this procedure was not followed, one of the flowmeter manometers or the differential manometer of the porometer were liable to blow out. No damage to the leaf-chamber seals resulted from the sudden release of the 12-cm. built-up pressure as there was sufficient volume in the humidifiers to absorb it. Other details of procedure have been described in the preceding sections.

THE 'LIGHT EXPERIMENT' (13 September to 3 October 1950)

1. *Design of the experiment.* As mentioned in the Introduction (p. 2) the preliminary experiments of Heath and Milthorpe (1950) gave an indication of a direct effect of light upon stomatal movement in the absence of external carbon dioxide. The present experiment was intended to check this finding

using a 'through' flow of CO_2-free air to reduce the uncertainty as to the carbon-dioxide concentrations in the substomatal cavities.

The formal design of the experiment consisted of two 3×3 Latin squares with the treatments, 800, 270, and 90 f.c. light intensity occurring once in each row and once in each column in the usual manner and thus with 6-fold replication. Each row of a square represented a different leaf, which was treated with the three light intensities in one days' experimentation. The columns represented the first, second, and third equilibrium periods during the day, but since it was necessary to wait for an equilibrium under one light intensity before changing to another the times showed a considerable scatter. The mean times were 12.05 p.m., 3.03 p.m., and 6.35 p.m. (G.M.T.).

Preliminary experiments had indicated the existence of a diurnal rhythm of stomatal movement in wheat, and although much of its effect should have been eliminated from the treatment means by the Latin square design, it was decided to include on each leaf a second 'control' pair of chambers receiving a constant light intensity of 270 f.c. It was hoped that by using the differences 'experimental' - 'control' as data a more precise evaluation of the treatment effect would be achieved. The arrangement of the control and experimental chambers is described above (p. 6). In the second Latin square the positions of control and experimental chambers were reversed so that any effect of the relative positions on the leaf might be included in the statistical analysis.

During the overnight period preceding each day's experimentation both the control and experimental areas of the leaf received 270 f.c. and the intervening 1·7 cm. received 800 f.c.

The data have been both analysed and presented as $\log_{10}$ (resistance) without correction for area of chamber of stomatal numbers. Such a correction, being entirely 'between leaves', would not affect the light or diurnal effects, which were found 'within leaves'.

2. *Results*. Analysis of variance was first carried out on the values for $\log_{10}$ (resistance) from the experimental chambers only and a significant light effect was found ($P < 0·05$), most of the effect being between 90 and 270 f.c. When the analysis was repeated using 'experimental' - 'control' values the significance of the light effect was much *reduced* and now only just passed the P 0·05 level. This suggested that the control leaf area was affected by the light changes in the experimental area and the 'control' values were therefore analysed separately. A significant light effect was found here also ($P < 0·05$), though much smaller in magnitude than in the 'experimental' area. The analyses of variance for the three sets of data are shown in Table I and the light effects are shown graphically in Fig. 2. Correction for areas and stomatal numbers would affect the position but not the shapes of the curves.

DISCUSSION

The significant light effect found for the 'experimental' chambers would seem to show the existence in wheat of a light effect upon stomatal movement

TABLE I

'Light Experiment', 1950. Analyses of variance on values of $\log_{10}$ *(resistance). (2 Latin squares, each 3×3)*

Variation	Degrees of freedom	Experimental		Experimental-control		Control	
		Variance	F*	Variance	F*	Variance	F*
Total	17	—	—	—	—	—	—
Treatment (light)	2	1·114	6·89	0·533	4·65	0·108	5·47
Rows† (leaves)	4	0·458	2·83	0·383	3·33	0·414	20·5
Columns (order)	2	0·154	—	0·039	—	0·044	2·20
Squares (position)	1	1·110	2·42	0·510	1·33	0·115	—
Residue (error)	8	0·162	—	0·115	—	0·020	—

* Significant levels of variance ratio (*F*): For 2 and 8 degrees of freedom: 4·46 at *P* 0·05; 8·65 at *P* 0·01. For 4 and 8 degrees of freedom: 3·84 at *P* 0·05; 14·39 at *P* 0·001. For 1 and 4 degrees of freedom: 7·71 at *P* 0·05.

† Error for squares (position).

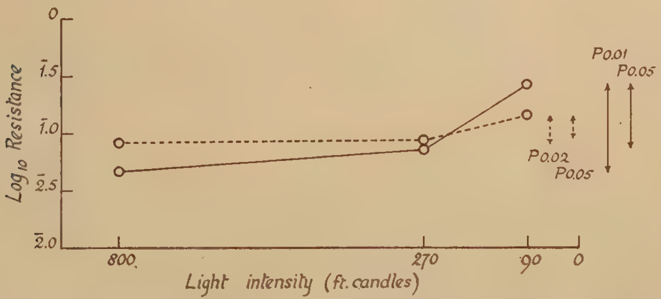


FIG. 2. Effect of light intensity on stomatal movement in carbon dioxide-free air. Continuous line: experimental area, light varied. Dotted line: control area at 270 f.c. throughout, showing light effect transmitted from experimental area across a 1·7-cm. 'light barrier' at 800 f.c.

independent of that operating through the intercellular space atmosphere, and as already mentioned (p. 2) the simplest interpretation of this would be a *direct* effect upon the guard cells themselves. Heath and Milthorpe (1950) found a light effect with zero *or* 0·01 per cent. carbon dioxide in the air supplied to the leaf surface, although in their experiments its significance could not be tested, and over this range the stomata showed no response to external carbon dioxide. These observations have been fully confirmed in work to be described in the next paper of this series (Heath and Russell, in the press). Since the apparent direct light effect is found under conditions in which the stomata are not measurably affected by changes of external carbon dioxide (between 0·01 and 0·00 per cent.), and therefore presumably of internal carbon dioxide in the guard cells, it apparently operates by some other mechanism than the reduction of internal carbon dioxide content, such as might occur, for example, with photosynthesis in the guard cells.

The conclusion that the observed light effect is independent of control by the mesophyll and intercellular space atmosphere might be invalid if the rate of flow of carbon-dioxide-free air through the stomata was very inadequate, for then at low light intensity the carbon dioxide concentration in the substomatal cavities might rise above the 0·01 per cent. which gives maximal opening. An estimate may be made on the basis of an assumed rate of light respiration. Gabrielsen's (1948) results for *Sambucus* indicate that light respiration approximates to that in darkness and is of the order of 0·5 mg. CO_2 dm.^{2} hr. If the same rate is assumed for wheat and the area concerned is taken as that enclosed by the leaf chamber, viz. 1·11 cm.^{2}, the weight of respiratory carbon dioxide produced would be 0·0056 mg. hr. In 250 c.c. of air this would give a concentration of 0·0011 per cent. This represents the value which would be obtained by uniform mixing; actually much of the carbon dioxide would be produced in the mesophyll and therefore the concentration at the guard cells would be considerably lower. It thus appears almost certain that a virtually carbon dioxide-free atmosphere was maintained in contact with the guard cell surfaces, or at least a concentration far below 0·01 per cent., and the postulated direct effect of light may be accepted as independent of the intercellular space carbon dioxide concentration on these grounds. Control by some other component of the intercellular space atmosphere is a possibility, but seems improbable with a 'through' flow of air sweeping the substomatal cavities.

The apparent direct effect of light might in fact be due, wholly or in part, to an indirect effect transmitted not through the intercellular space system but from cell to cell, either from the epidermal cells or the more remote mesophyll. Such an effect seems unlikely to be transmitted by carbon dioxide gradients, for the subsidiary and other epidermal cells are devoid of chloroplasts in 'Charter' wheat, while the diffusion paths to the mesophyll are very long and the resistance to aqueous diffusion very high; furthermore, it has been argued above that the effect does not operate by reduction of the carbon dioxide in the guard cells. There is, however, some evidence of an indirect effect of light transmitted from one part of the leaf to another in the results obtained with the 'control' chambers. The trustworthiness of this evidence should now be considered.

Tests in which the 'experimental' and 'control' chambers were illuminated as in the actual experiment and the light intensity for the former was changed between 800 and 90 f.c. (or even zero) were carried out with a photo-electric cell and meter. These indicated a change in intensity in the 'control' chambers due to internal reflection of light along the perspex block of less than 1 per cent. or, say, 2 f.c. No transference of light along the leaf could be detected. The stomatal response found for the 'experimental' leaf area in terms of $\log_{10}R_{10.000}$ was 0·0035 per f.c. between 90 and 270 f.c. or 0·00036 per f.c. between 270 and 800 f.c.; in the 'control' area the mean response was 0·245 when the treatment in the 'experimental' area varied between 90 and 800 f.c. Taking the figure of 0·0035 per f.c. this response would require 70 f.c., and this is clearly a gross underestimate, for instead of the slope between 90 and 270 f.c. the much less steep slope *at* 270 f.c. should really be used. It seems therefore that the apparent transmission of a light effect cannot have been an artefact due to light transference along the perspex or the leaf.

The statistical evidence for the transmission effect although significant is by no means overwhelming: in the analysis on the 'Control' values the significance of the 'Light' effect fell short of P 0·01 although better than P 0·05; the mean log resistance for 90 f.c. was higher than in both the other treatments for five out of six leaves and higher than in 800 f.c. for all six; 270 and 800 f.c. showed no consistent difference, although in the 'experimental' values the log resistance for 270 f.c. was higher than for 800 f.c. in every leaf. Even there, however, the mean difference was much smaller than that from 90 to 270 f.c. and not significant by the t test. The 'transmission effect' should be accepted for the present, though perhaps with some caution pending further confirmation.

It seems most improbable that the light effect can have been transmitted from the 'experimental' to the 'control' area by means of the carbon dioxide or indeed any other constituent of the intercellular space atmosphere, for the intercellular space system within and adjacent to each pair of chambers was being swept with 250 c.c./hour of carbon dioxide-free air, and diffusion *into* the control or the experimental area would have to take place against an air-stream under the washers with a flow rate approaching 125 c.c./hour. Nor does it seem likely that an appreciable continuous gradient of carbon dioxide concentration from cell to cell can have existed across the intervening 1·7 cm. of leaf at a constant high light intensity of 800 f.c. This last argument might be expected to apply to gradients of almost any other kind due to change caused by difference of light intensity, and in order to persist across such a high light barrier the change must be one produced by low light intensity (such as 90 f.c.) but not reversed at all rapidly by high light intensity. It appears premature to speculate as to the nature, chemical or perhaps electrical, of such a change. A re-balancing of turgor in the epidermis following light-induced turgor changes in the guard cells of the experimental area would not appear to provide a satisfactory explanation—this would be expected to have only a temporary effect lasting until the guard cells in the control area had once more come to equilibrium with the epidermal cells, when the turgor difference between guard cells and epidermal cells, and hence the stomatal posture, would be the same as before. In any case, such effects would be expected to be negligibly small owing to the small volume of the guard cells compared with the epidermal cells.

Transmission by some chemical or electrical stimulus not travelling in the intercellular space system thus seems the least improbable mechanism. Williams (1948) obtained indications that 'heat shock' stimuli causing stomatal closure were transmitted along the phloem in the leaf of *Pelargonium*. In that paper he also describes apparently independent stomatal responses to light and dark within two porometer cups on the same leaf, but these cups were in all cases separated by main veins, unlike those in the present experiments. Moreover, in his experiments, one of the cups was always in light while the other was in darkness or vice versa; thus although they certainly showed stomata opening and closure under the appropriate conditions, it was impossible to say that the rate of movement and the final level reached were not affected by the conditions in the other cup. Williams (1948*a*) later himself acknowledged on other grounds, which need not concern us here, that these experiments did not in fact demonstrate independent stomatal behaviour in the two cups.

In conclusion it may be said that the experiment described in the present paper tends to confirm the suggestion of a light effect independent of carbon dioxide seen in Heath and Milthorpe's (1950) data, but leaves undecided the question of whether it is entirely an indirect effect transmitted from epidermal or mesophyll cells by some unspecified agent travelling from cell to cell, or whether it is in part due to a direct light response in the guard cells themselves of the kind postulated by those authors.

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A Comparison of the Effects of 3-Indolylacetic Acid and 3-Indolylacetonitrile on the Development of Sporelings of Marsilea in Aseptic Culture

A. ALLSOPP

Dept. of Cryptogamic Botany, University of Manchester

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SUMMARY

Sporelings of *Marsilea Drummondii* were grown aseptically on a basic medium of mineral nutrients with addition of 2 per cent. glucose and a range of concentrations of 3-indolylacetic acid and 3-indolylacetonitrile.

Apart from certain changes in the root system the acid had no pronounced effect on the cultures when present at 0·01, 0·1, and 1·0 mg./l.; but at 10 mg./l. it was markedly toxic, the sporelings remaining small and malformed. In constrast, the nitrile supported very vigorous growth at 10 mg./l., and the internodes and petioles of the plants became strikingly elongated, although there was no increase in the area of the leaf lamina.

Examination of the epidermal cells of internode and petiole indicated that in this layer at least the elongation was not merely a result of cellular extension, but was accompanied or caused by increased transverse division of the cells.

The significance of the results is briefly discussed.

INTRODUCTION

THE isolation from cabbage of the highly active growth hormone 3-indolylacetonitrile (Jones *et al.*, 1952) together with the further accounts of its biological properties by Bentley and Housley (1952) and Bentley and Bickle (1952) has shown that this substance may be of great significance as a naturally occurring plant hormone. In the present work the effect of the nitrile on the development of sporelings of the water-fern *Marsilea* has been investigated. *Marsilea* was selected for study, in the first place as an example of a pteridophyte, and secondly because previous work (Allsopp, 1952) had shown that sporelings of this plant when growing aseptically in liquid culture are particularly sensitive and uniform in their response to the action of various growth substances.

MATERIALS AND METHODS

The experimental work was carried out on *Marsilea Drummondii* A. Br., an Australian species.

Sterile cultures were prepared following the technique described in the earlier work. The sporelings were again grown separately in glass tubes

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containing 10 ml. of the basic inorganic medium, with 2 per cent. glucose (A. R.) present as a carbon source. The growth substances, 3-indolylacetic acid (IAA) or 3-indolylacetonitrile (IAN) were added before autoclaving, which was carried out at 110° C. for 20 minutes. It was clear from the experimental results that the nitrile remained stable under this treatment and that any hydrolysis could not exceed a maximum of 1 per cent.

Sporelings were grown in media containing IAA at concentrations of 0·01, 0·1, 1·0, and 10 mg./l.; and IAN at 0·1, 1·0, 10, and 100 mg./l. Other sporelings were grown on the glucose medium without added growth substance. The sporelings all originated from a single sporocarp which yielded over a hundred embryos. The embryos were transferred to the culture tubes within 24 hours of the original opening of the sporocarp. Detailed examination was made of eight replicate cultures from each medium.

EXPERIMENTAL RESULTS

Differences between the sporelings on the various media were already apparent after a few days. Some of the differences became more marked with longer exposure, while others became less obvious. The condition of the cultures after 11 weeks is illustrated by representative plants in Fig. 1.

The results obtained with IAA were very similar to those described in the earlier work (Allsopp, 1952) but are reported here briefly for comparison with the effects of IAN. With 0·01 mg./l. IAA the sporelings were little different from those on the 2 per cent. glucose medium (Fig. 1*a* and *b*). At 0·1 mg./l the first few roots were inhibited in length, but produced numerous laterals, although this effect soon disappeared and later roots remained unbranched like those in the normal glucose medium (Fig. 1*c*). With 1 mg./l. IAA the early roots were again short and had many laterals. They became dark and swollen as a result of expansion and loosening of the cortical cells. There was again a fairly rapid recovery to the normal condition (Fig. 1*d*). For the three lower concentrations of IAA there was a slight increase in growth rate (as measured by overall size and increase in leaf number) with each increase in concentration of the acid, but at 10 mg./l. IAA, the sporelings were extremely stunted and malformed with almost complete root inhibition and irregular hypertrophy of the rhizome cortex to form a callus-like mass (Fig. 1*e*).

With IAN the effects were different both qualitatively and quantitatively. At 0·1 and 1 mg./l., in contrast with the results obtained with IAA, there was no root inhibition or increased tendency towards the formation of lateral roots. At 1 mg./l., however, the root-hairs were particularly luxuriant. With 0·1 mg./l. IAN the amount of growth was only slightly greater than on the glucose medium (Fig. 1*f*), but at 1 mg./l. (Fig. 1*g*) in addition to an increase in the rate of leaf formation there was a greater elongation of petioles and internodes, although the differences between these sporelings and those on the glucose medium were still not very pronounced. The effect of 10 mg./l. IAN was outstanding (Fig. 1*h*). From the figure it is apparent that the internodes of the rhizome and the petioles have undergone great elongation, although the

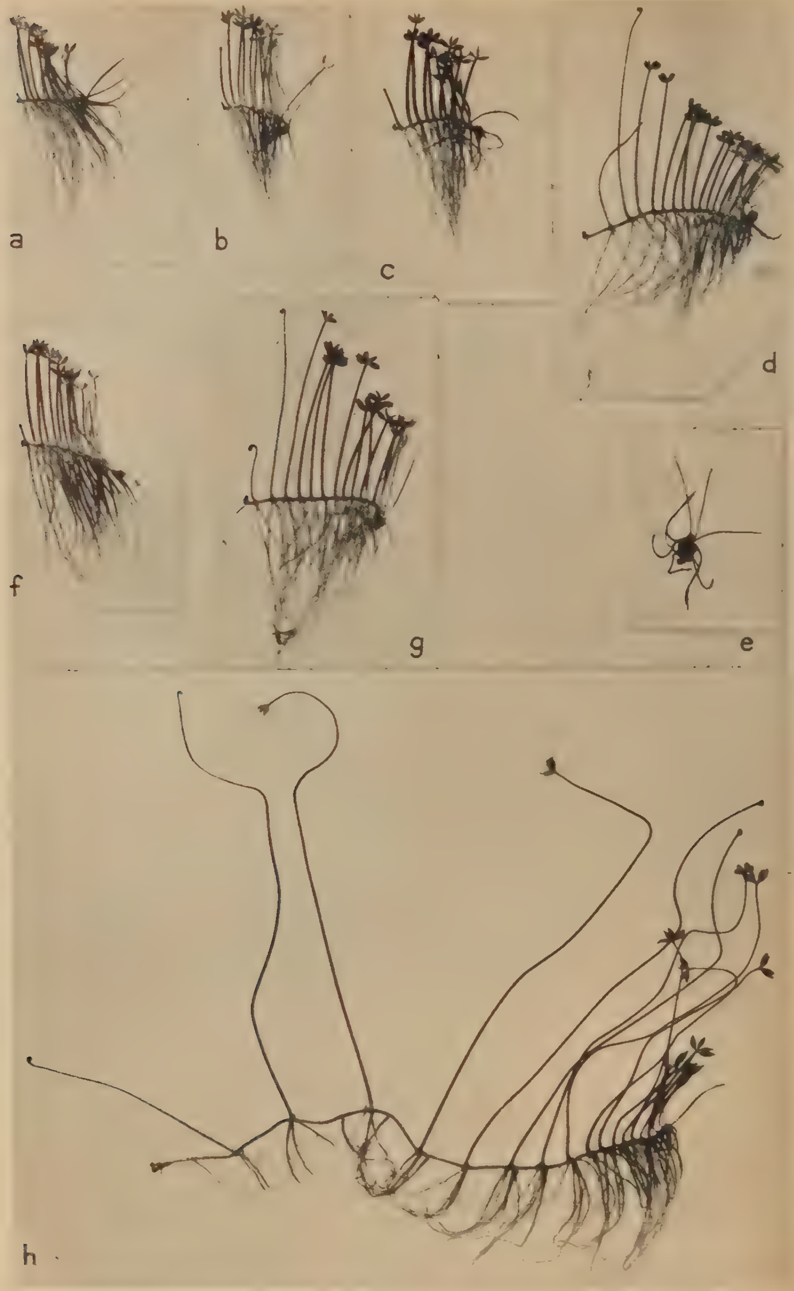

FIG. 1. *M. Drummondii*. Sporelings after 11 weeks of growth in a 2% glucose medium containing (*a*) glucose only, (*b*) 0·01 mg./l. IAA, (*c*) 0·1 mg./l. IAA, (*d*) 1·0 mg./l. IAA, (*e*) 10 mg./l. IAA, (*f*) 0·1 mg./l. IAN, (*g*) 1·0 mg./l. IAN, (*h*) 10 mg./l. IAN. (All nat. size.)

laminae of the leaves are practically unaffected. The roots bear many laterals and even the early roots showed no more growth inhibition than was found in 0·1 mg./l. IAA. There was no indication of the type of root injury found in 1 mg./l. IAA. At 100 mg./l. IAN development was completely suppressed and the embryos soon died.

The features apparent from general observation of the cultures were confirmed by a series of measurements, which are summarized in Table I. The measurements were made on the 12th leaf and 12th internode, since more recent parts had not attained complete development in all the cultures of the glucose series. In the other three series studied, the differences shown in the table were even more pronounced in the later formed regions.

TABLE I

Effect of various concentrations of indoleacetic acid and indoleacetonitrile on sporelings of Marsilea Drummondii. Mean values from 8 cultures per treatment

	Basic medium (2% glucose) only	Basic medium +1 mg./l. IAA	Basic medium +1 mg./l. IAN	Basic medium +10 mg./l. IAN
Number of leaves per plant after 11 weeks of growth . .	14·1	17·0	16·1	18·4
Length in mm. of internode 12	0·75	1·18	1·60	2·70
Length in mm. of petiole of leaf 12	10·45	11·31	16·06	32·13
Length in mm. of lamina of leaf 12	1·64	1·81	1·68	2·01
Ratio of lamina to petiole length in leaf 12	1:6·4	1:6·3	1:9·6	1:16·0

The effects of IAA and IAN on the morphology of the leaf are illustrated in Fig. 2, which presents outline drawings of the 12th leaf from seven of the sporelings shown in Fig. 1. It is evident from this figure, and also from the measurements given in Table I, that neither IAA at 0·01, 0·1, or 1 mg./l. nor IAN at 0·1 mg./l. have any appreciable effect on the size or morphology of the leaf. With IAN at 1 mg./l. there is some extension of the petiole, which becomes greatly elongated in IAN at 10 mg./l. As shown in the figure, the area of the lamina remains approximately constant regardless of the length of the petiole. The different ratios of lamina:petiole length for various concentration of the two growth substances are set out in Table I. Table I also shows that in 1 mg./l. IAN there is greater extension of the 12th internode than in 1 mg./l. IAA. This elongation is considerably more pronounced in 10 mg./l. IAN.

In view of the marked elongation of petiole and internode in IAN, an examination was made of the epidermal cells with the object of ascertaining whether in this layer at least the great elongation of the region could be referred to cellular extension. The lower epidermis of the leaf lamina was also examined. After decolorization in formalin-acetic-alcohol fixative, the lamina could be studied microscopically without any further treatment, while the petiole and internode were first split lengthwise to increase their transparency. Drawings were made with a camera lucida.

Drawings of the lower epidermis were made from the leaves illustrated in Fig. 2 *a*, *d*, *f*, and *g*, and are shown in Fig. 2 *h*, *i*, *j*, and *k* respectively. The figures demonstrate that the added growth substances have not resulted in any increase in the size of the cells of the lower epidermis of the lamina. The epidermis of the petioles of the same leaves and of the 12th internodes of the sporelings shown in Fig. 1 *a*, *d*, *g*, and *h*, are illustrated in Fig. 3. From the figure it is evident that in the glucose medium, and in 1 mg./l. IAA and IAN, differences in petiole or internode length are not accompanied by any marked differences in the size of the epidermal cells. In 10 mg./l. IAN, however, there is some cellular elongation, accompanied by a slight narrowing of the cells, but this relatively slight extension falls far short of accounting for the

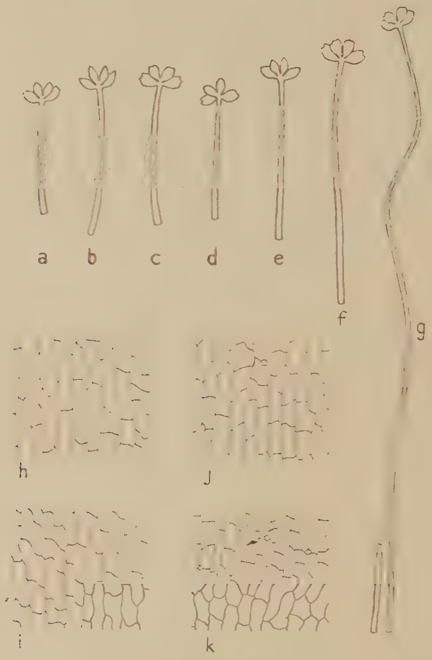

FIG. 2. *M. Drummondii*. *a–d*. The 12th leaf of the sporelings illustrated in Fig. 1, *a–d* and *f–h*, i.e. from glucose only, from 0·01, 0·1, and 1·0, mg./l. IAA and from 0·1, 1·0, and 10 mg./l. IAN respectively. (All × 1·7.) *h–k*. Cells from the lower epidermis of the laminae of the leaves shown in *a*, *d*, *f*, and *g*, i.e. from glucose only, form 1·0 mg./l. IAA and from 1·0 mg./l. and 10 mg./l. IAN respectively. (× 112.)

great length of petiole and internode and there must consequently have been a considerable increase in the number of transverse divisions of the epidermal cells.

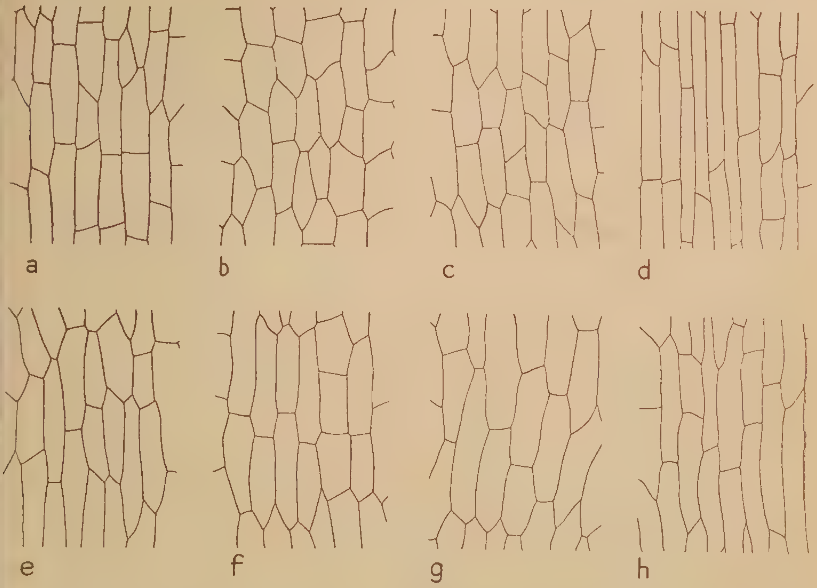

FIG. 3. *M. Drummondii. a–d.* Epidermal cells from petioles of leaves illustrated in Fig. 2 *a, d, f,* and *g,* i.e. from glucose only, from 1·0 mg./l. IAA, and from 1·0 mg./l. and 10 mg./l. IAN respectively. *e–h.* Cells from epidermis of 12th internode of the plants whose petiole cells are illustrated in Figs. *a–d.* (× 150.) In Figs. *a–d* and *e–h* the cells are elongated in a direction approximately parallel to the longitudinal axis of petiole and rhizome respectively.

DISCUSSION

From the experimental results it is clear that 3-indolylacetonitrile has a marked effect on the morphology of sporelings of *Marsilea.* The effect is different both qualitatively and quantitatively from the effect of IAA. The most striking response to the nitrile is the marked elongation of both petiole and internode. It is evident from examination of the epidermal cells of these regions that the elongation is not due purely to cell extension, but is accompanied by a considerable increase in cell number. Since there is no increase in the diameter of the affected parts, there must have been an increase in the number of transverse divisions of the cells of the meristematic region, but whether this was a cause or consequence of the elongation of the internodes or petioles was not determined. The extension of the petioles and internodes is relatively slight with IAA, and in this connexion it should be mentioned that Bentley and Housley (1952) found that IAN is considerably more active than IAA in the *Avena* straight growth test.

There is also a great difference between IAN and IAA in their effect on root growth. Bentley and Bickle (1952) found that IAN is approximately 100 times less active than IAA in inhibiting the root growth of cress. In the present work it was similarly found that the extent of root inhibition in *Marsilea* was no greater with IAN at 10 mg./l. than with IAA at 0·1 mg./l.

The lower toxicity of the nitrile is another important difference from IAA. The contrast between the large vigorous sporelings from 10 mg./l. IAN (Fig. 1, *h*) and the stunted malformed plants from 10 mg./l. IAA (Fig. 1, *e*) could hardly be greater. With IAA there was injury to the roots at 1 mg./l., but with IAN no such injury was detected even at 10 mg./l.

The greater stability of the nitrile may prove to be an important property of this hormone. It is known that IAA is very rapidly inactivated by coleoptile sections (Larsen and Bonde, 1953), while the restricted duration of the production of abnormal roots by *Marsilea* sporelings in a culture medium originally containing 1 mg./l. IAA indicates that here also there has been rapid inactivation of the IAA. In view of this inactivation of IAA, the greater activity of the nitrile in certain tests does not rule out the possibility that it exerts its effects as a result of hydrolysis to IAA. Externally supplied IAA might well be inactivated during its entry into the tissues and thus never reach an optimum concentration. An enzyme-controlled production of IAA from the nitrile might also obviate the toxic effects of high concentrations of IAA.

The low toxicity of the nitrile and the parallel responses of such widely separated plants as *Marsilea*, and those used by Bentley and her co-workers, indicate that IAN may be an important naturally occurring plant hormone. Quite recently Bennet-Clark and Kefford (1953) have indeed detected the nitrile, by paper chromatography, in a considerable range of plant materials. Bentley and Bickle (1952) found that the nitrile was active in certain tests affecting cell-division in meristematic tissues. But although the nitrile was originally extracted from cabbage leaves, in the present work it had no noticeable effect on the leaf lamina of *Marsilea*. In this respect it resembles other growth substances previously tested (Thimann, 1948; Went, 1951; Allsopp, 1952) and is therefore not the long sought mesophyll growth factor or 'phyllocaline'.

I am indebted to Professor C. W. Wardlaw, for his helpful suggestions and encouragement, and to Mr. E. Ashby for the photographs in Fig. 1. I am grateful to Dr. J. A. Bentley for a sample of pure 3-indolylacetonitrile, which was originally synthesized by Dr. G. F. Smith in the Chemistry Department, University of Manchester.

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The Submicroscopic Structure of Pectin in Collenchyma Cell-Walls

P. A. ROELOFSEN AND D. R. KREGER

Laboratory of Technical Botany and X-ray Department of the Laboratory of Technical Physics, Technical University, Delft, Netherlands

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RECENTLY Preston (1952) has commented in this journal on our conclusions concerning the condition of pectic substances in the collenchyma cell-walls of *Petasites vulgaris* (Roelofsen and Kreger, 1951). This comment appears to be somewhat discouraging. Meanwhile, with full acknowledgement of Dr. Preston's experience of this material, we cannot agree with his remarks. We would like, therefore, to make some notes in our defence.

PECTIN SKELETONS

1. Preston first argues that our method of cellulose extraction with a solution of cupric hydroxide in ammonia is inadequate, and he states: 'Under these conditions it seems inevitable that oxidation will occur and the cellulose will be partially reprecipitated as cellulose II.'

We have imitated the conditions of the extractions, using cotton instead of collenchyma bundles, but we could not find even traces of cellulose in the centrifuged remains. We did not claim complete absence of cellulose. If a portion of pectin skeleton—and not 'small parts' of it, as Preston without any reason suggests—was dissolved by solvents which do not dissolve cellulose, only traces of cellulose sphaerocrystals and some lignified spiral thickenings would be detected in the centrifuged remains. It was much too minute a quantity to have any effect at all on the optical or the X-ray observations. Moreover, optical anisotropy and oriented X-ray interferences could never be due to this material.

2. With regard to the demonstration of the form-double-refraction curve of the pectin skeletons Preston condemns the use of many imbibition liquids instead of mixtures of only two.

Purposely we only used homogeneous liquids instead of mixtures of two, since preferential absorption of one of the two might occur. Moreover, satisfactory results had already been obtained with similar homogeneous liquids on pectin fibres by previous authors (e.g. Wührman and Pilnik, 1945). Meanwhile, our main purpose was not to produce a smooth curve, but to leave no doubt on the occurrence of negative intrinsic double refraction, since this would prove the presence of crystalline oriented pectin. Because oriented molecular absorption of some liquids has been described in literature, a great number of imbibition liquids were used.

3. Preston's argument is that in the pectin skeletons 'the presence of a *high degree* of axial orientation is not proved. This lack of orientation', he proceeds, 'could be due, as Roelofsen and Kreger point out, to a shrinkage of the specimens during cellulose extraction.'

Thus Preston suggests first that we would claim to have proved a *high degree* of orientation, whereas immediately thereafter he states—and quite rightly—that in fact we are pointing out why there is a *lack* of orientation. This seems rather mysterious. Of course we have often used the term 'axially oriented' for the pectin in pectin skeletons, but, taking into account that we repeatedly stressed the low degree of axial orientation, it seems evident that the qualification 'axial' was only meant to indicate the direction of the predominant orientation.

THE NATIVE CELL-WALL

4. Preston argues that 'observation of a single arc corresponding to a spacing present in many substances besides pectin, including native cellulose, can hardly be accepted as demonstrating even the presence of crystalline pectic compounds'. We wish to remark that, according to Preston and Duckworth (1946), these collenchyma cell-walls consist largely of a 'cellulose component' and 44 per cent. of 'pectic substances'. Although the analysis was not made in detail, there appeared no evidence of any other kind of substance being present in quantity. Taking also into account our general knowledge of the cell-wall constituents of higher plants and the fact that the cuprammonium-extracted collenchyma rapidly dissolves in typical pectin solvents and, finally, the results of the optical and X-ray observations on these skeletons, it seems permitted to leave those 'many substances' out of consideration.

It merely remains for us to establish, therefore, whether the meridional arc could be due to cellulose or to pectin. The spacing corresponding to the arc is 4·23 Å. The nearest cellulose spacings of sufficient intensity to be considered are 3·92 Å (002) and 4·39 Å (021). Therefore the spacing does not point in the direction of cellulose and, moreover, pectin-free cellulose skeletons do not yield any arc at all in the meridian with this spacing; but pectin skeletons do.

This would seem evidence in favour of the assumption that the arc in question in the pattern of untreated bundles must be due to the 44 per cent. pectic substances.

In addition, the cellulose (021) arcs are clearly discernable on the original of our Fig. 4, though, admittedly, we have seen that in some copies this detail has not withstood reproduction.

5. Preston proceeds: 'in addition the lateral 3·9 Å arcs, which are spread into a complete circle, overlap in this position. The consequent danger implicit in the interpretation of arcs occurring in this position has already been pointed out (Preston, 1946). There is abundant evidence to deny this view, supplied partly by Preston himself in his joint paper on these collenchyma bundles (Preston and Duckworth, l.c.). It is established in that paper that the equatorial cellulose arcs in the pattern of unstretched collenchyma are

confined to an angle as small as about 10°. Therefore the danger of overlap i the meridian may be excluded. This danger would exist indeed if the angl were much wider as in the case referred to by Preston, viz., the wood o certain conifer species with sufficiently flattened spirals in the tracheides.

6. Then Preston objects: 'Secondly the pectin-free bundle *only* had bee slightly stretched. This normally has the effect of separating the (021) arc and clearing up the meridian, before any visual change occurs in the equa torial arcs.' In other words, the stretching would have caused improve orientation of the cellulose and 'The absence of a meridional arc attributabl to pectin might therefore be due merely to stretching'.

We wish to remark that the extraction of pectin had caused shrinkage o the collenchyma bundles and consequently an increased angular dispersion o the cellulose crystallites. As is apparent from Fig. 5 in our paper, the stretch was not sufficient to restore the original degree of orientation, for the angula spreading of the lateral cellulose arcs is clearly much higher than in Fig. 4 New prints of these diagrams, with the contrasts adapted, demonstrate thi difference. [Costs prevent their inclusion here; they have been seen by Dr Preston: *Editor*.]

Thus it is clear that the degree of cellulose orientation in the stretche pectin-free bundle is not higher, as Preston suggests, but lower than in th untreated bundle. Consequently, if the meridional arc on the pattern of th untreated bundle were due to overlapping of cellulose interferences, it shoul be more pronounced still in that of the extracted bundle instead of havin vanished. The absence of the meridional arc in the latter case is, therefore again strong evidence that it must be attributed to pectin.

7. As regards Preston's feeling that 'in particular the evidence fails t demonstrate any *high degree* of axial orientation' we must admit that thi qualification, used in two places, taken *literally*, is exaggerated. We shoul have better stressed that 'high' has been used here mainly as an opposite o 'low' to differentiate between the degree of orientation in the native collenchyma on one hand and the pectin skeletons on the other. Meanwhile, a les critical reader may have understood this from the context on p. 342: 'On th other hand, a high degree . . ., etc.' Nevertheless, we could have used mor adequately the qualification 'rather high', which would indicate the differenc equally well and would have been in better harmony with the actual conditions.

R. D. PRESTON

Department of Botany, The University, Leeds

My criticism of the work of Roelofsen and Kreger was naturally made o the basis of their published account and was intended mainly to call attentio to the fact that the paper *as written* did not establish a *high degree* of axia orientation in the pectic component as claimed by these authors. The above supplement to the original statements makes clear the two following points (*a*) A meridional arc corresponding to molecular planes of spacing 4·23 Å is clearly present in their original photographs and, since the walls contain more than 40 per cent. of pectic materials, this furnishes strong presumptive evidence of the presence, in untreated collenchyma cells of *Petasites*, of pectic compounds with some degree of crystallinity. (*b*) The existence of this meridional arc demonstrates a tendency towards axial orientation which may be called a *rather high* (but surely not an unqualified *high*) degree of orientation. This is to my mind a point of major importance since in the search for the mechanism of polysaccharide orientation it is important to know which molecular species can be well oriented. The above remarks of Roelofsen and Kreger make it clear that the degree of orientation is not high, so that we are now in agreement.

In the question of the fusion of arcs along the meridian, I referred to my later work on wood rather than to the earlier publication on *Petasites* because such fusions were referred to in the wood papers only. In *Petasites* collenchyma the 3·9 Å arc is commonly spread into a ring, so that when the 021 arcs are fused along the meridian the impression can be given of an arc at about 4·2 Å. This was the impression given by the published photographs of Roelofsen and Kreger. Their original photographs do show, however, a separate arc at about 4·2 Å.

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The Enzymic Decarboxylation of γ-methyleneglutamic Acid by Plant Extracts

L. FOWDEN

Department of Botany, University College, Gower St., London, W.C.1

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SUMMARY

γ-Methyleneglutamic acid, an acidic amino-acid isolated from groundnut plants, was decarboxylated by enzymes present in extracts of *Capsicum* fruits, barley roots, and tulip leaves, and also by intact cells of *Clostridium welchii S.R. 12*. The amino-acid was attacked in a similar manner to, but in all cases at a slower rate than, *l*-glutamic acid. The nature of the enzyme responsible for the decarboxylation of γ-methyleneglutamic acid was further investigated using preparations from barley roots (which do not contain the amino-acid) and from tulip leaves (in which the amino-acid is normally present, together with larger amounts of its amide form, γ-methyleneglutamine). The effects of pH, inhibitors, and partial heat denaturation upon the enzyme systems present in the barley and tulip extracts indicated that a single enzyme was responsible for the decarboxylation of both *l*-glutamic acid and γ-methyleneglutamic acid. Although the *Cl. welchii* rapidly deamidated and then decarboxylated *l*-glutamine, γ-methyleneglutamine was not attacked by the organism.

INTRODUCTION

γ-METHYLENEGLUTAMIC acid and its amide form, γ-methyleneglutamine, are normal constituents of groundnut plants, and both compounds have been isolated in pure form from the vegetative parts of the plants (Done and Fowden, 1952). In this paper, following a terminology used in earlier papers, γ-methyleneglutamine will be subsequently referred to as I, γ-methyleneglutamic acid as II, and a synthetically prepared γ-methylglutamic acid as III. II has been found to participate in many reactions similar to those that are now recognized as metabolic pathways for glutamic acid, an amino-acid that is structurally very closely related to II; but, whereas glutamic acid has a general occurrence in plant tissues, II appears to be present only in a limited number of the species so far examined.

One type of reaction in which this similarity is seen between the two amino-acids is their enzymic decarboxylation at the α-COOH grouping by plant extracts, and Fowden and Done (1953*a*) have shown that II is quantitatively converted to carbon dioxide and α-methylene-γ-aminobutyric acid (IV) by extracts of *Capsicum* fruits and barley roots. On the basis of these earlier experiments, II is considered to be the *l*-stereoisomer. It is interesting to note that barley roots have also been shown to contain an enzyme active in the decarboxylation of *l*-glutamic acid (Beevers, 1951). Since II appears to be

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absent from these tissues, no specific enzyme responsible for the decarboxylation of II would be expected to be present, and decarboxylation is probably catalysed by the glutamic acid decarboxylase. Extracts of tulip leaves also bring about decarboxylation of II and *l*-glutamic acid, and it appears that some II and considerably larger amounts of I are present in these leaves. Here two possibilities arise: the enzymic complement of the leaves may be such that either two distinct decarboxylase enzymes are present, each acting in a specific manner on its own substrate; or the plant may make one enzyme suffice to carry out both decarboxylations. This paper presents evidence supporting the latter mechanism.

METHODS AND MATERIALS

The plant extracts used as sources of decarboxylase activity were prepared in the following ways. In all cases the extracts were finally buffered using citric acid-Na_2HPO_4 mixtures according to McIlvain's standards.

Capsicum sp. The fruits of a West African *Capsicum* species (red pepper) were used. They were finely chopped and then ground using a pestle and mortar with a minimum amount of concentrated pH 5·6 buffer and a little washed sand until a smooth paste was obtained. The paste was squeezed through muslin and the filtrate centrifuged for 5 minutes at 1,000*g*. The supernatant extract so obtained was used in the manometric experiments.

Barley roots. Pioneer barley seedlings, grown in washed sand without the addition of any nutrients in a greenhouse maintained at 70°, were carefully harvested after 15 days' growth. The roots were removed, washed well, and finally rinsed in distilled water. In order to obtain a more active enzyme preparation, the extracts were concentrated using the $(NH_4)_2SO_4$ precipitation technique of Beevers (1951). The roots were first ground in the same way as the *Capsicum* fruits, but with distilled water replacing the buffer solution. Five grammes of $(NH_4)_2SO_4$ were then added to each 10 ml. of supernatant extract obtained, and after all the salt had dissolved, the precipitated proteins were removed by centrifugation. The supernatant liquid was discarded and the residue redissolved in a small volume of buffer of appropriate pH. The solution was used in the later manometric experiments.

Tulip leaves. Mature leaves were harvested and ground with buffer of appropriate pH (1 ml. of buffer for each 4 g. fresh weight of leaves) and a small amount of washed sand. The further extraction procedure was as with *Capsicum* fruits above.

Clostridium welchii. The S.R. 12 strain, N.C.T.C. 6784 (kindly supplied by Dr. E. F. Gale, F.R.S.), was grown on a medium containing 2 per cent. glucose, 3 per cent. Difco Bacto-Casitone, and 1 per cent. minced heart-muscle for 18 hours at 25°. After straining the culture through fine muslin to remove the pieces of meat, the cells were harvested by centrifugation at 500*g* for 5 minutes, and then twice washed with distilled water. The cells obtained from 1 l. of medium were finally resuspended in 25 ml. of the appropriate pH 0·2 M. acetate buffer.

Manometry. The conventional Warburg technique was employed to follow the production of CO_2 and hence the course of the decarboxylation. Two millilitres of the extract or suspension of the organism were placed in the main chambers of the Warburg flasks, whilst 0·5 ml. of the substrate solutions were tipped from the side-arms at zero time. In the flasks serving as controls 0·5 ml. of distilled water were tipped from the side-arms. Preliminary experiments were performed with 0·2 ml. of a concentrated KOH solution in the centre-wells of duplicate flasks to ensure that in all instances the only gas change, additional to that of the control flasks, after tipping the substrates was due to the evolution of CO_2. All experiments were performed at 25° under anaerobic conditions, the manometers and flasks being flushed with oxygen-free nitrogen for 15 minutes before transference to the bath. Fifteen minutes were allowed for temperature equilibration before the substrates were tipped.

RESULTS

The rates of decarboxylation of *l*-glutamic acid and II were compared under similar conditions using each of the four sources of decarboxylase activity. The pH was adjusted to 5·6 when extracts of *Capsicum* fruits, barley roots, and tulip leaves were used, since this pH was near to the optimum determined for the glutamic decarboxylase activity found in a wide variety of plant materials (Okunuki, 1937; Schales and Schales, 1946*a*, 1946*b*; Schales, Mims, and Schales, 1946; Beevers, 1951). In the experiments using *Cl. welchii* a pH of 4·5, recommended as the optimum (Gale, 1945), was used. Fig. 1 (*a–d*) illustrates the courses of the decarboxylation reactions.

In general the rate of decarboxylation of each substrate fell steadily with time, indicating that the amounts of the substrates supplied were probably insufficient to saturate the enzymes. The rates of decarboxylation were therefore obtained by constructing tangents to the carbon dioxide output curves at zero time. It was found that the rates so obtained for *l*-glutamic acid were considerably greater than those for an equimolar amount of II, being 2·1 times faster when the barley-root extract was used, 2·4 times greater using tulip extracts, 2·8 times faster for *Capsicum* extracts, and 3·7 times faster for *Cl. welchii*. Manometric measurements were terminated when at least 60 per cent. of the II used had been decarboxylated; during these times the carbon dioxide evolved indicated that nearly complete decarboxylation of *l*-glutamic acid had occurred in all cases. In contrast to these results obtained for II, its reduced form III (prepared synthetically) did not appear to be significantly decarboxylated by the barley root extracts and only at a very low rate by *Cl. welchii*. III was not tested with extracts of *Capsicum* or tulips.

As stated earlier, chromatographic analysis of extracts of barley roots failed to reveal II as a normal constituent, but its presence in tulip leaves was indicated. The further experiments described using extracts of barley roots and tulip leaves were designed to test the hypothesis that II and *l*-glutamic acid were in both instances decarboxylated by a single decarboxylase whose substrate specificity was not absolute. In these experiments the concentrations of the substrate solutions (0·5 ml.) placed in the side arms were 0·03 M. with respect to the *l*-forms.

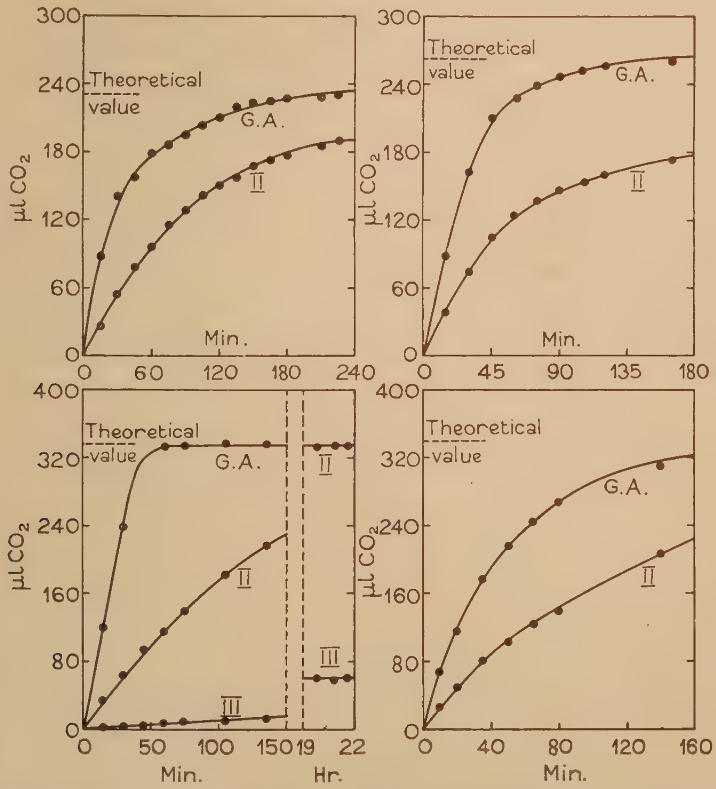

FIG. 1. Rate of decarboxylation of *l*-glutamic acid (G.A.), γ-methyleneglutamic acid (II), and γ-methylglutamic acid (III) by (*a*) an extract of the fruits of a *Capsicum* sp. (red pepper) at pH 5·6, (*b*) an extract of barley roots at pH 5·6, (*c*) *Clostridium welchii S.R. 12* at pH 4·5, and (*d*) an extract of tulip leaves at pH 5·6.

Effect of pH. In these experiments the barley root extracts used were prepared by redissolving the whole of the ammonium sulphate precipitated proteins in a suitable volume of distilled water, and then buffering aliquots (three volumes) of this solution at six different pH values in the range 4·0–6·8 by the addition of one volume of a concentrated phosphate-citric acid buffer solution of appropriate pH. Tulip extracts were prepared by the addition of one volume of buffer to three volumes of centrifuged juice expressed from macerated leaves. Extracts were prepared at five different pH values within the range 4·5–6·5. The rates of decarboxylation of *l*-glutamic acid and II at zero time were obtained for each preparation from the appropriate carbon dioxide output curves, and the pH/activity relationships for the decarboxylation of the two substrates are shown in Fig. 2 (*a* and *b*). It can be seen that the curves for *l*-glutamic acid and II are of very similar shape when either extracts of barley or tulips were used. The optimum pH for the decarboxylation reactions was about 5·9 for the barley enzyme and 5·8 for the tulip preparations. Table I shows that the ratio of the decarboxylation rate of *l*-glutamic acid to that of II remains virtually constant for each type of extract regardless of the pH used.

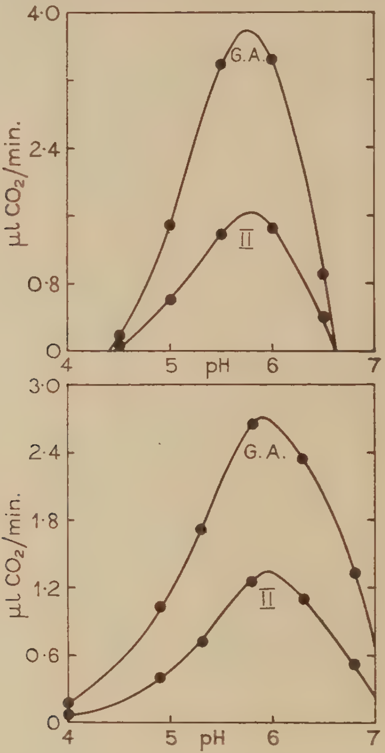

FIG. 2. The effect of pH on the rates of decarboxylation of *l*-glutamic acid (G.A.) and γ-methyleneglutamic acid (II) by extracts of (*a*) tulip leaves and (*b*) barley roots.

Effect of inhibitors. The effect of inhibitors on the rates of decarboxylation of II and *l*-glutamic acid at pH 5·6 were determined. The detailed results obtained using semicarbazide as an inhibitor are given here. The semicarbazide was added to the enzyme preparations in the main chambers of the Warburg flasks before the addition of the substrates at zero time. The amounts used were such that the inhibitor concentrations in the final reaction mixtures were

TABLE I

Showing the ratios between the rates of decarboxylation of l-glutamic acid (G.A.) and γ-methyleneglutamic acid (II) at various pH values using extracts of barley roots and tulip leaves

Barley roots		Tulip leaves	
pH	$\frac{\text{Rate for G.A.}}{\text{Rate for II}}$	pH	$\frac{\text{Rate for G.A.}}{\text{Rate for II}}$
4·0	2·5	4·5	2·5
4·9	2·5	5·0	2·5
5·3	2·4	5·5	2·4
5·8	2·1	6·0	2·4
6·3	2·2	6·5	2·4
6·8	2·5	—	—

0·001 M. and 0·0025 M. In Table II the rates of decarboxylation of *l*-glutamic acid and II by barley and tulip preparations in the presence of semicarbazide are compared with the rates of the corresponding reactions in the absence of the inhibitor. All rates are evaluated at zero time.

TABLE II

Showing the effect of semicarbazide as an inhibitor on the rates of decarboxylation of II and l-glutamic acid

Concentration of semicarbazide. Molar		Rate for *l*-glutamic acid. μl.CO_2/min.	Rate as a percentage of uninhibited rate	Rate for II. μl.CO_2/min.	Rate as a percentage of uninhibited rate	$\frac{\text{Rate for } l\text{-glutamic acid}}{\text{Rate for II}}$
Barley roots	0	2·47	100	1·18	100	2·1
	0·001	1·83	74	0·91	77	2·0
	0·0025	0·91	37	0·43	36	2·1
Tulip leaves	0	4·04	100	1·48	100	2·7
	0·001	3·12	77	1·20	81	2·6
	0·0025	1·66	41	0·64	43	2·6

Effect of heat on the enzymes. In this series of experiments barley and tulip preparations buffered at pH 5·6 were divided into three parts which were (*a*) not further treated, (*b*) maintained at 55° for 5 minutes, and (*c*) maintained at 55° for 10 minutes before use in the manometric experiments. The rates of decarboxylation of II and *l*-glutamic acid were determined at zero time using each enzyme preparation, and the results obtained are recorded in Table III.

TABLE III

Showing the effect of heating the plant tissue extracts on the rates of decarboxylation of II and l-glutamic acid

Time of heating at 55° Min.		Rate for *l*-glutamic acid. μl.CO_2/min.	Rate as a percentage of unheated enzyme rate	Rate for II. μl.CO_2/min.	Rate as a percentage of unheated enzyme rate	$\frac{\text{Rate for } l\text{-glutamic acid}}{\text{Rate for II}}$
Barley roots	0	2·55	100	1·21	100	2·1
	5	2·16	85	1·06	85	2·0
	10	1·89	74	0·94	74	2·0
Tulip leaves	0	3·64	100	1·46	100	2·5
	5	2·58	71	0·99	68	2·6

Enzyme specificity. Beevers (1951) has reported that the decarboxylase pr sent in barley roots did not produce any measurable effect when *l*-aspart acid was supplied as substrate. The enzyme was also ineffective for thos amino-acids which are attacked by the various bacterial decarboxylase Working with enzyme preparations buffered at pH 5·6, the findings of Beever with regard to *l*-aspartic acid have been confirmed, and the range of possibl substrates has been extended to include other compounds closely related t glutamic acid. No measurable amounts of carbon dioxide were produce from III, *dl*-α-amino-adipic acid, or *dl*-α-amino-pimelic acid in 3 hours; i the same time *l*-glutamic acid was nearly completely decarboxylated.

Beevers (1951) showed that the barley-root enzyme preparation was unabl to decarboxylate *l*-glutamine. It has not been possible to determine whethe I, the amide form of II, could be decarboxylated by barley roots due to th extremely small amounts of I available. Since *Cl. welchii S.R. 12* has bee used as an assay organism not only for *l*-glutamic acid but also for *l*-glutamin (Gale, 1945; Krebs, 1948), the ability of this organism to deamidate and the decarboxylate I was tested. The experiment was performed in 0·2 M. p 4·9 acetate buffer as recommended by Krebs (1948). *l*-glutamine was used a a comparison substance. Although the theoretical carbon dioxide output from *l*-glutamine was recorded in 60 minutes, no carbon dioxide was produced from an equimolar amount of I in 200 minutes.

DISCUSSION

Each of the four plant materials investigated contained a decarboxylase capable of rapidly removing carbon dioxide from II, although this amino-acid was not a normal constituent of either barley roots or *Cl. welchii*. Although the properties of the enzyme systems from the different sources varied slightly (at pH 5·6 *l*-glutamic acid was decarboxylated 2·1 times faster than II using the barley-root extracts, whereas the ratio was increased to 2·3 in the case of tulip-leaf extracts and to 2·8 with the *Capsicum* extracts), the rate of decarboxylation of *l*-glutamic acid was always greater than that found for II, irrespective of the enzyme preparation or conditions used. These facts suggested that a single enzyme may have been responsible for the removal of carbon dioxide from both substrates.

Other criteria that must be satisfied before the single enzyme hypothesis can be regarded as likely are (*a*) that the activity of the enzyme should change with alterations in the working pH in a similar manner for both substrates, (*b*) that inhibitors should affect the enzyme's activity towards both substrates to an equal degree, and (*c*) that partial heat denaturation of the enzyme should produce a similar decrease in its activity towards both substrates. The results obtained (Fig. 2 (*a* and *b*) and Tables II and III) using enzyme preparations from both barley roots and tulip leaves conform to these requirements. During the course of the experiments many different preparations from both barley and tulips were made, but on all occasions the relative rates of decarboxylation of *l*-glutamic acid and II remained the same. Had two separate enzymes been present, it is very unlikely that all the preparations used would have contained them in the same relative proportions. The conclusion has therefore been drawn that a single enzyme was responsible for the decarboxylation of *l*-glutamic acid and II in all the materials examined.

The optimum pH of about 5·9 observed for the decarboxylation of *l*-glutamic acid by barley root preparations was higher than that of 5·2 determined by Beevers (1951), who used a different strain of barley seedlings, but was within the range of the optimum pH values determined for a number of other plant species.

Fig. 1 (*c*) shows that when II was added to suspensions of *Cl. welchii* under the conditions normally used for the manometric assay of *l*-glutamic acid by the method of Gale (1945), some 40 per cent. of the II added had been decarboxylated before the theoretical carbon dioxide had been evolved from an equimolar amount of *l*-glutamic acid. *Cl. welchii* would then be unsuitable for the assay of *l*-glutamic acid in amino-acid mixtures when II was also present in appreciable amounts. II has not so far been detected in animal tissues and is present only in a limited number of plant materials, and so its interference in the assay technique will also be limited. Since *Cl. welchii* cannot deamidate I (and thus decarboxylate the II formed), the assay of *l*-glutamine by the organism would be unaffected by the presence of I. A method of estimating the amounts of glutamine, asparagine, and I in mixtures of all three amides could probably be based on the fact that only glutamine would be assayed by *Cl. welchii*, whilst I, like glutamine, reacts as 'unstable amide', leaving asparagine to be estimated as 'stable amide'.

Schales and Schales (1946*b*) used the powder obtained by freeze-drying aqueous extracts of squash tissue as a source of a specific decarboxylase for the estimation of *l*-glutamic acid. Although the effect of such enzyme preparations on II have not been investigated, it would appear doubtful whether the specificity of the squash enzyme for *l*-glutamate is absolute in view of the results obtained for the other plant decarboxylases studied here.

Certain interesting conclusions can be drawn from the results recorded earlier regarding the structural features that must be present in amino-acids before they can act as substrate molecules. The decarboxylase present in *Cl. welchii* requires that a suitable substrate should contain an unbranched five-carbon atom chain, with —COOH and —NH_2 groups arranged as in *l*-glutamic acid, but the substitution by certain other groupings of the hydrogen atoms on the β and γ carbon atoms is permissible. β-hydroxyglutamic acid (Gale and Epps, 1944) and II (both hydrogen atoms on the γ-carbon atom are substituted by a methylene grouping in the latter compound) were attacked by the organism. The measured decarboxylation rates were lower than those for *l*-glutamic acid, probably because the larger groupings replacing the hydrogen atoms on the main chains of the molecules sterically hindered the approach of the molecules during the formation of the enzyme-substrate complex. III was attacked very much more slowly than II, but it is difficult to draw conclusions from this observation since III can exist in four stereoisomeric forms, which were probably all present in the synthetic material used. Two of these isomers would have a *d*-configuration at the α-carbon atom and so would not be attacked. Both of the other two isomers having an *l*-configuration at the α-carbon atom may have been attacked, but probably at different rates according to the different orientations of the γ-methyl group in relation to the rest of the molecule. Similar conclusions can be drawn for the substrate requirements of the decarboxylase present in barley roots, excepting that in this case III appeared to be unattacked. This difference would seem to indicate that small structural differences occur in the enzymes themselves. The deamidase of *Cl. welchii* was more exacting in its substrate requirement than the decarboxylases, and the substitution of a methylene group for the two hydrogen atoms on the γ-carbon atom of glutamine gave a compound (I) which was not attacked by the enzyme.

A further comparison can be made between the decarboxylases investigated here and the enzymes responsible for the transference of the α-amino-group of II and III to α-ketoglutarate, which was present in groundnut plant tissues. Although the replacement of the methylene group of II by a methyl group and a hydrogen atom to give III produced a marked change in the rates of decarboxylation of the *l*-forms of the amino-acids, the same structural change in the substrate molecule was without effect on the rates of the transamination processes (Fowden and Done, 1953*b*).

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Absorption of Nitrogen, Phosphorus, and Potassium from Nutrient Sprays by Leaves[1]

G. N. THORNE

Rothamsted Experimental Station, Harpenden, Herts.

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SUMMARY

Barley, Brussels sprout, French bean, tomato, and sugar-beet plants grown in soil in pots and sprayed, usually daily, for several weeks, with nutrient solutions containing nitrogen, phosphorus, potassium, and a spreader, with precautions to prevent the spray solution falling on the soil, had higher nutrient contents and dry weights than control plants sprayed with water and spreader only. Increase in nutrient content occurred with high or low levels of nutrient supply to the roots and was approximately proportional to the concentration of spray and to the frequency of spraying.

The nitrogen content of sugar-beet plants was increased equally by spraying with solutions supplying ammonium sulphate, calcium nitrate, or urea in equivalent concentrations.

Nutrient uptake from solutions sprayed on leaves influenced uptake by the roots so that the additional amounts of nutrient contained in sprayed plants may be greater or smaller than the amount absorbed from the spray by the leaves.

INTRODUCTION

It has been known for some time that micronutrients can be absorbed through the leaves of plants; deficiencies of these elements in the field can often be corrected more easily and certainly by supplying them in solutions sprayed on the leaves than by soil application (Wallace and Ogilvie, 1941; Wallace and Jones, 1942). Application of major nutrients by sprays to agricultural and horticultural crops, with which the present work is concerned, has been used intermittently (mainly in the U.S.A.) over the past 30 or 40 years. Recently there has been a renewed interest in this method of applying fertilizers, because the increased use of overhead irrigation in market gardens, and of spraying machinery for controlling diseases and pests on farms and in orchards, makes it practicable. If fertilizers are supplied in a larger volume of spray solution than the leaves can retain, some of the solution will fall to the soil, and therefore any beneficial effect from the nutrients supplied will not necessarily imply that they have entered the plant through the leaves. Spraying with large volumes of solutions may merely be a convenient method of fertilizer distribution.

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[1] The work described in this paper formed part of a thesis for the degree of Ph.D. in the University of London.

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Differences between the effects of fertilizer applied in irrigation water and as solid, claimed by Secrett (1949) and obtained in a Rothamsted experiment on sugar-beet (Rothamsted Experimental Station, Results of field experiments, 1949), were probably due to the variation in time of application of the fertilizer and not to absorption of nutrients by the leaves. Fruit trees frequently benefit from spraying with urea solutions, and Fisher (1952) concluded from 4 years' experiments on apple trees that urea sprays gave yields similar to, or greater than, those obtained from comparable soil dressings. The yield of Laxton's Superb apple bushes of high nitrogen status was unaffected by spraying with a 1 per cent. urea solution (Bould and Tolhurst, 1951), and the yield and nitrogen content of tomatoes in field plots was increased equally by urea sprays or by equivalent soil dressings of nitrogenous fertilizers (Montelaro, Hall, and Jamison, 1952). Thus it is not clear from the literature what factors determine whether field crops will benefit from fertilizers applied as sprays, nor whether the beneficial effects can be accounted for by nutrients absorbed through the leaves.

The most reliable evidence that plants can absorb nutrients through their leaves comes not from field experiments but from pot experiments, often using radioactive tracers, where solutions were applied to the leaves in such a way that none of the applied nutrients could be absorbed through the roots. Lewis (1936) sprayed the leaves of phosphate-deficient lettuce plants with a solution containing potassium sulphate, potassium phosphate, and sodium nitrate, and found that phosphorus, but not the nitrogen and potassium content of the treated plants, after careful washing, was significantly greater than that of the unsprayed control plants. Cook and Boynton (1952) sprayed individual leaves of apple whips with urea solutions and found that nitrogen was absorbed within 2 hours. Rodney (1952) found increased growth in apple following nitrogenous sprays. Silberstein and Wittwer (1951) detected entry of ^{32}P into the plant from the leaves in the cases of maize, tomato, and bean seedlings. Wittwer and Lundahl (1951) found that French beans absorbed ^{32}P quicker through the leaves than through the roots. Tukey and others (1952) found that compounds containing radioactive potassium, phosphorus, or carbon were absorbed by the stems and branches as well as the leaves of apple-trees.

The aim of the following experiments was to investigate the effects of nutrient sprays supplying nitrogen, phosphorus, and potassium on the nutrient content and growth of a variety of species mainly of agricultural crops. In addition there was an experiment on sugar-beet using sprays supplying nitrogen only.

EXPERIMENTAL TECHNIQUE

Method of growing plants. Plants were grown singly in pots of Rothamsted soil mixed with 20 per cent. by weight of sand, except for barley where there were 8 plants per pot. The soil used for Expts. 1, 5, and 8 was of a higher nutrient status than for the other experiments, and in Expt. 5 peat was also added. Six grammes of $(NH_4)_2SO_4$ and 3·3 g. K_2HPO_4 per pot were added to the soil at sowing for the high fertilizer treatment of Expt. 6. In the case of the similar treatment in Expt. 5. 6 g. of nitro-chalk and 12 g. each of superphosphate and potassium sulphate were applied during the season to each pot; the other plants received no fertilizer. All plants were watered with 'de-salted' tap water prepared by electro-osmosis, avoiding free drainage.

All plants were grown during the summer out of doors, except that in bad weather and at night they were brought into a glasshouse. All experiments were of randomized block design with 4 to 8 replicates, and the plants of each block were selected to be as similar in size as possible just before spraying started.

Method of spraying. The spray solutions used for all the experiments, except 8, were made from a mixture of $NH_4H_2PO_4$, NH_4NO_3, and KNO_3 in the ratio 1:3·1:5·3, supplying nitrogen, phosphorus, and potassium in the ratio 7:1:7·3, approximately the same as that found in plant tissues. Solutions supplying nitrogen, but not phosphorus or potassium, were used in Expt. 8. The spray used in Expts. 1, 2, 6, and 7 contained 2·69 g. N, 0·38 g. P, and 2·78 g. K per litre. This was diluted to two-thirds of this concentration for Expt. 3 and to half for Expts. 4 and 5. When there were two concentrations of spray, the dilute spray was half the concentration given above. There were 3·50 g. N per litre in the spray of Expt. 8. The sprays were chosen by tests made before each experiment, to be as concentrated as was possible without scorching the leaves, but in spite of this, slight damage sometimes occurred. The spray solutions were made up with glass-distilled water in Expts. 1, 5, and 8 and with de-salted water in the remainder, and all contained 0·05 per cent. sulphonated lorol as a spreader. The control treatment was always spraying with water plus spreader. The nutrient solutions were applied as a finely atomized spray with a paint-gun operated by compressed air. Each pot had a cover of waterproof fabric fitted round the stem of the plant to prevent spray or drip from the leaves falling on the soil. The spraying was sufficient to wet the upper surfaces of the leaves thoroughly without causing much run off, and some spray also reached the under surfaces. A portable shield was used to prevent spray reaching plants other than the one being sprayed.

Spraying was started when the plants were well established, about 6 weeks after sowing for sugar-beet, and continued throughout the growing period, so that plants were usually sprayed for about 14 weeks between early June and mid-September. In most experiments spraying was 'daily', which in practice was six times a week.

The day before harvesting all plants were well washed with a spray of water to remove nutrients deposited on the surface of the leaves.

Growth measurements. At harvest the plants were divided into leaves, stems, roots, fruit, &c., and each part weighed, dried at 100° C., and weighed again. The roots were washed with a jet of water to remove soil and then dried, either with a cloth or by centrifuging in a perforated basket head, to remove surface moisture before weighing. The dried plant material was kept for nitrogen, phosphorus, and potassium determinations. Leaf area per plant was determined from the fresh weight of the leaves and estimates of the area: weight ratio obtained by weighing and counting a sample of disks of known area punched from the mass of detached leaves (Watson and Watson, 1952).

Estimation of nutrient content of plant tissue. For nitrogen, phosphorus, and potassium determinations the plant material was finely ground and redried at 100° C. Nitrogen was determined by the micro-Kjeldahl method. Phosphorus was estimated colorimetrically with acid molybdate and stannous chloride. Potassium and calcium determinations were done on a flame-photometer. When the sugar content of sugar-beet roots was required, samples were taken from the roots at harvest and extracted in boiling 95 per cent. alcohol. The sugar was estimated in the alcohol extract as invert sugar by the method of van der Plank (1936).

DESCRIPTION OF EXPERIMENTS AND RESULTS

In the following account the term 'nutrient content' is used for the weight of a nutrient in a single entire plant. The difference between the nutrient content of plants sprayed with nutrient solution and of plants sprayed with water will be called *nutrient uptake* from the spray. Whether this increased nutrient content really measures the amount of nutrient absorption from the spray will be considered in the discussion.

The following varieties were used: barley, mildew resistant variety B. 2039 from Plant Breeding Institute, Cambridge; Brussels sprouts, Dobbie's Favourite; French bean, Canadian Wonder; tomato, Harbinger; sugar-beet, Kleinwanzleben E.

(*a*) *Experiments with sprays supplying nitrogen, phosphorus, and potassium*

Expt. 1. *Barley* (Table I). Spraying with complete nutrient solution caused increases in nitrogen, phosphorus, and potassium contents and dry weight. Higher nutrient contents and dry weights of grain, straw, and chaff, but not of roots, contributed to this effect and the increased grain weight was entirely due to the extra tillers of the plants sprayed with nutrient solution because there was no effect of treatment on 1,000-grain weight or number of grains per ear. Plants sprayed with nutrient solution were darker green and ripened later than those sprayed with water.

Expt. 2. *Brussels sprouts* (Table I). Spraying with concentrated nutrient solution significantly increased the content of nitrogen, phosphorus, and potassium and dry weight. The dilute spray gave smaller increases in nutrient content but a similar increase in dry weight. Spraying increased the dry weight of leaf, stem, and root.

Expt. 3. *French bean* (Table I). Unfortunately a seed infection caused uneven germination and growth in this experiment. The dilute spray had no detectable effect on nutrient content. The concentrated spray significantly increased phosphorus content, and there were indications that the nitrogen and potassium contents and the dry weights of leaf, stem, pods, and root were also increased.

TABLE I

Expts. 1, 2, 3. Effect of spraying with complete nutrient solution on nutrient content and dry weight of barley, brussels sprouts, french beans and tomato. g. per pot (8 plants of barley, 1 plant of the others)

			Nutrient content			Dry
		Treatment	N	P	K	weight
Expt. 1. Barley		Control	1·51	0·171	1·85	111
		Spray	1·99	0·198	2·35	128
		L.S.D.	0·13	0·020	0·26	9
Expt. 2. Brussels sprouts		Control	0·66	0·138	0·88	69
		Dilute spray	0·71	0·167	1·12	88
		Concentrated spray	1·20	0·182	1·19	89
		L.S.D.	0·22	0·024	0·20	11
Expt. 3. French bean		Control	1·72	0·166	0·83	68
		Dilute spray	1·46	0·176	0·90	59
		Concentrated spray	2·11	0·209	1·22	82
		L.S.D.	0·44	0·037	0·44	26
Expt. 4. Tomato		Control	0·76	0·155	0·98	57
		Spray	0·90	0·174	1·23	64
		L.S.D.	0·12	0·019	0·11	5
Expt. 5. Tomato	low fertilizer	Control	1·32	0·229	2·47	88
		Spray	1·45	0·224	2·85	89
	high fertilizer	Control	3·57	0·496	5·71	174
		Spray	3·90	0·520	6·26	180
		L.S.D.	0·38	0·048	0·38	16

Expt. 4. Tomato (Table I). The foliage of all the plants was chlorotic and the fruit showed the symptom known as 'hard back', associated with potash deficiency. Spraying with a complete nutrient solution from 7 June to 14 August increased the nutrient content of leaves, stem, and fruit but did not suppress the deficiency symptom in leaves and fruit and caused only small increases in dry weight. The plants were inclined to wilt and root growth was poor. This was probably due to unsuitable conditions in the narrow glazed pots and may have accounted for the lack of growth response to nutrients absorbed from the spray.

Expt. 5. Tomato (Table I). The plants were much larger than in Expt. 4. Addition of fertilizer approximately doubled the nutrient content and dry weight, and spraying during June to September with the same solution as in Expt. 4 had no effect on dry weight. The increases in nutrient content due to spraying were trivial compared with those produced by the fertilizer application; the largest and only significant one was for potassium.

Expt. 6. Sugar-beet (Figs. 1 and 2). This experiment was similar in design to Expt. 2, except that the sprays were applied to plants growing with two levels of nutrient supply to the roots and that, in order to follow the course of nutrient uptake from soil and spray throughout the growing period, samples were taken 7 times during the season. The first harvest was made just before spraying started and the others 2, 4, 7, 10, 14, and 18 weeks later.

Nutrient content was increased throughout the experiment by fertilizer added to the soil and from July onwards by nutrient sprays (Fig. 1). The concentrated spray gave increases in nutrient content slightly more than twice those obtained with the dilute spray, both with and without fertilizer added to the soil, and the increases from both sprays were greater with added fertilizer.

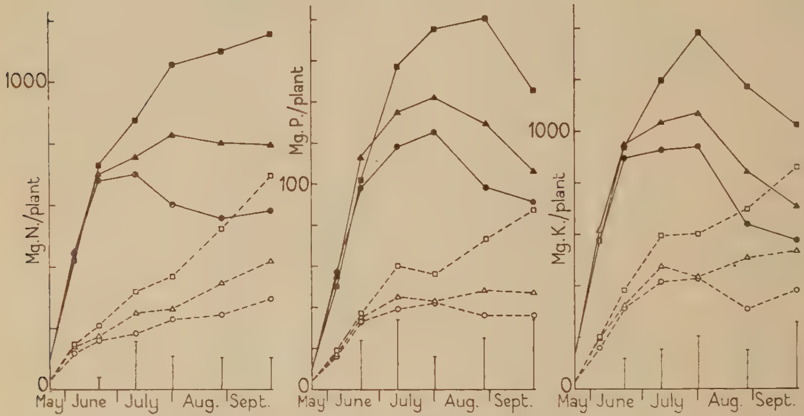

FIG. 1. Expt. 6. Effect of nutrient sprays on nutrient content of sugar-beet. Least significant differences ($P = 0{\cdot}05$) are shown at the foot of each graph. □- -□ daily spraying with concentrated spray, △- -△ daily spraying with dilute spray, ○- -○ daily spraying with water; no fertilizer added to soil. ■—■ daily spraying with concentrated spray, ▲—▲ daily spraying with dilute spray, ●—● daily spraying with water; fertilizer added to soil.

The fall in phosphorus and in potassium content per plant for all spray treatments with fertilizer added to the soil after mid-July was due, at least in part, to loss of nutrients in dead leaves which were not included in the samples. For nitrogen the fall was smaller or absent either because of greater translocation of nitrogen than of phosphorus and potassium from the dead leaves, or because nitrogen continued to be absorbed relatively faster than phosphorus and potassium from the spray.

Nutrient sprays caused increases in dry weight approximately parallel to the increases in nutrient content (Fig. 2). Treatment had no effect on percentage of fresh weight of sugar in the roots at the final harvest, so that there were increases in sugar per root parallel with the increases in root weight. Leaf area per plant was increased at all harvests by fertilizers added to the soil and by nutrient sprays after the middle of June; the largest effect was from concentrated spray. Changes in the nutrient content and dry weight of leaves, petiole, and roots contributed to the increased total nutrient content and dry weight obtained from nutrient sprays and fertilizer added to the soil.

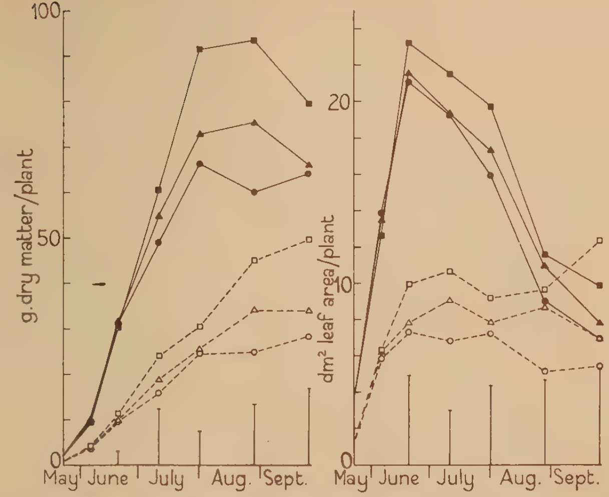

FIG. 2. Expt. 6. Effect of nutrient sprays on dry weight and leaf area of sugar-beet. Least significant differences ($P = 0{\cdot}05$) are given at the foot of each graph. □– –□ daily spraying with concentrated spray, △– –△ daily spraying with dilute spray, ○– –○ daily spraying with water; no fertilizer added to soil. ■—■ daily spraying with concentrated spray, ▲—▲ daily spraying with dilute spray, ●—● daily spraying with water; fertilizer added to soil.

Expt. 7. Sugar-beet (Table II). This experiment was intended to investigate the effect of frequency of spraying and also to determine whether daily spraying with water, the control treatment in other experiments, had any effect. The treatments were:

O Unsprayed (2 plants per block).
W Sprayed daily with water (2 plants per block).
1 Sprayed once a week with nutrient solution and not sprayed on the other days.
2 Sprayed twice a week (Tues. and Fri.) with nutrient solution and not sprayed on the other days.
6 Sprayed daily with nutrient solution for 17 weeks (7 June–4 Oct.).
6*a* Sprayed daily with nutrient solution for 8 weeks (7 June–2 Aug.).
6*b* Sprayed daily with nutrient solution for the 9 weeks following treatment 6*a* (2 Aug.–4 Oct.).

Table II shows that nutrient content and dry weight were unaffected by spraying with water or spraying only once a week with nutrient solution, but were increased significantly by the other treatments. This increase was greatest for treatment 6 and least for treatment 2. Increases in nutrient content and weight were found in all parts of the plant, and as there was no treatment effect on the percentage sugar composition of the roots, the yield of sugar per root was therefore directly proportional to the root weight.

TABLE II

Expt. 7. Effect of time and frequency of spraying with a complete nutrient solution on the nutrient content and dry weight of sugar-beet. Grams per plant

Treatment	Nutrient content N	P	K	Dry weight
O	0·56	0·090	0·55	63
W	0·57	0·097	0·58	69
1	0·56	0·079	0·54	56
2	0·75	0·115	0·69	81
6	1·15	0·168	1·18	98
6*a*	0·89	0·121	0·70	76
6*b*	0·87	0·114	0·80	65
L.S.D. (*a*)	0·09	0·024	0·09	11
,, (*b*)	0·11	0·029	0·11	14
,, (*c*)	0·12	0·034	0·12	16

(*a*) L.S.D. ($P = 0·05$) between treatments O and W.
(*b*) ,, ,, ,, ,, O, W, and the rest
(*c*) ,, ,, ,, ,, 1, 2, 6, 6*a*, 6*b*.

(*b*) *Sprays supplying nitrogen*

Expt. 8. Sugar-beet (Table III). In all experiments using a complete NPK spray nitrogen was supplied as a mixture of ammonium and nitrate ions. In order to compare ammonium and nitrate ions and urea as sources of nitrogen in sprays, plants were sprayed daily throughout the summer with 0·125 M. solutions of ammonium sulphate, calcium nitrate, urea, or with water as the control treatment. Calcium nitrate was used to minimize any effect that cations absorbed from the nitrate spray might have on plant growth. As sodium can partly replace potassium in sugar-beet nutrition, sodium and potassium nitrates were undesirable salts.

All three nitrogen sprays increased the nitrogen content per plant by about 25 per cent. and the total dry weight was increased by nitrate and urea but not by ammonium sprays which, after about 6 weeks spraying, caused a marginal scorching of the older leaves. This damage remained until harvest, accounting for the small size of plants receiving this treatment. All parts of the plant contributed to the increases in nitrogen content and total dry weight obtained with the various treatments. There was an increase in sugar per root although sugar per cent. of fresh weight was very slightly reduced in the roots with high nitrogen contents. From these results it is not certain whether undamaged plants would absorb equal amounts of nitrogen from nitrate and ammonium sprays. In a preliminary experiment, where the leaves were undamaged, spraying small sugar-beet plants daily for 7 weeks with 0·125 M. ammonium sulphate solution increased the nitrogen content of the tops from

48 mg. to 62 mg., and spraying over the same period with 0·25 M. potassium nitrate increased the nitrogen content to 57 mg. It would appear, therefore, that nitrogen is readily absorbed from ammonium sulphate sprays by intact sugar-beet leaves.

Spraying with calcium nitrate solution increased the calcium content by 0·89 g. and the nitrogen content by 0·47 g., which is equivalent to 0·66 g. of calcium in calcium nitrate. Urea and nitrate sprays gave similar increases in dry weight, and with urea this was accompanied by absorption of 0·19 g. of calcium from the soil. If there was a similar uptake of calcium from the soil with the nitrate spray, 0·70 g. of calcium was absorbed from the spray. The similarity of this figure to the calculated value of 0·66 g. indicates that cation and anion were absorbed in approximately equivalent quantities from calcium nitrate solution sprayed on the leaves.

TABLE III

Expt. 8. Effect of various nitrogen-containing sprays on nitrogen and calcium content, dry weight, and sugar yield of roots of sugar-beet

Treatment	N per plant (g.)	Ca per plant (g.)	Dry weight per plant (g.)	Sucrose	
				g./root	% fresh weight
Control	1·23	1·22	125	56·0	16·3
Calcium nitrate	1·70	2·11	153	69·3	15·7
Ammonium sulphate	1·73	1·14	121	55·2	15·4
Urea	1·64	1·41	143	68·2	15·8
L.S.D.	0·14	—	17	9·4	1·5

DISCUSSION

There is no doubt that the differences between plants sprayed with nutrient solution and the controls were caused by the salts in the spray because the control plants were sprayed daily with water and spreader. Expt. 7 showed that the nutrient content and growth of the controls differed very little from those of unsprayed plants.

Expts. 1–7 show that uptake of nutrients from solutions sprayed on the leaves occurred with barley, Brussels sprouts, French beans, sugar-beet, and tomatoes, and was accompanied by increased growth in all except the second tomato experiment. Absorption of nutrients by the leaves was not restricted to plants with a very low nutrient supply to the roots. In Expt. 6 the increase with spraying in nutrient content per plant was greater when fertilizer was added to the soil (Fig. 1), and the large uptake of nitrogen from the sprays in Expt. 8 occurred in plants far from nitrogen deficient, as is evident from the nitrogen contents of the control plants (Table III).

The increase in nutrient content resulting from spraying was approximately proportional to the amount of nutrients supplied in the spray, when either the concentration of the sprays or the frequency of spraying were varied. In Expt. 6 doubling the concentration of the spray more than doubled the nutrient uptake (Fig. 1), and in Expt. 7 spraying six times a week gave more than three times the nutrient uptake obtained from spraying twice a week (Table II). The amount of spray solution retained by a plant depends on the leaf area, which is itself affected by nutrient supply. The plants receiving the concentrated spray in Expt. 6 had a greater leaf area than those receiving the dilute spray and therefore probably received more than twice the amount of nutrients. In the same way the plants of Expt. 7 sprayed six times a week had a greater leaf area during most of the experiment than those sprayed twice a week, and therefore retained more solution per plant at each spraying.

It is not clear whether the absorption of nutrients from sprays depends on the age of the plant. In Expt. 6 there appeared to be little uptake from the sprays after the end of August, whereas in Expt. 7 spraying after 2 August gave increases in nutrient content per plant similar to those obtained from spraying in June and July.

Although spraying increased plant dry weight, the increases were not closely related to the amount of nutrients absorbed from the sprays (e.g. Expts. 2 and 3). Some factor other than nutrient supply may have been limiting the growth of the Brussels sprouts but not of the tomatoes in Expt. 5, because fertilizer added to the soil caused a big increase in growth. The absence of a growth response to spraying by tomatoes was probably due to the small quantities of nutrients supplied by the dilute spray solution necessary to avoid scorching. Plants without fertilizer added to the soil absorbed only about one-tenth as much nitrogen and potassium from the spray as was taken up from fertilizer added to the soil, but spraying increased the dry weight by 1 g. per plant compared with the 86 g. per plant produced by the fertilizer application. In this experiment nutrients absorbed from spray were much less effective in increasing dry weight than were those absorbed from fertilizer added to the soil, whereas in Expt. 6 nutrients absorbed from spray or fertilizer gave similar increases in dry weight of sugar-beet (Figs. 1 and 2). The increases in dry weight obtained from sprays of different concentrations in Expt. 6 were approximately proportional to the amounts of nutrients absorbed, but the late spraying in Expt. 7 (Table III) caused no increase in dry weight although there was considerable uptake of nutrients from the spray. The distribution of nutrients and dry weight between tops and roots were similar when nutrients were absorbed by leaves or by roots.

Nitrogen, phosphorus, potassium, and calcium were all absorbed by leaves from sprays containing salts of these nutrients, but when a spray supplying several nutrients was used, they were not always absorbed in the same proportions as their concentrations in the spray solution. Expt. 8 (Table III) showed that nitrogen was absorbed equally easily from sprays when supplied as cation, anion, or an un-ionized compound.

It has so far been assumed that any increase in nutrient content resulting from spraying represents nutrient uptake by the leaves. There are several reasons why this may be unjustified. The washing of the leaves before harvest may be insufficient to remove a surface deposit, or, on the other hand, may leach nutrients from the tissues, and the estimate of uptake would be correspondingly either too high or too low. On the other hand, increased nutrient content of the roots of sprayed plants is good evidence of uptake by the leaves from the spray, because no spray solution was allowed to fall on the soil.

Increases in growth, caused by absorption of some constituents of a mixed spray, may lead to increases in nutrient content, not by absorption from the spray but by extra uptake by the roots, as, for example, in Expt. 8, where the calcium content of plants was increased by spraying with urea. Therefore, increases in the plant of all the nutrients in a mixed spray do not necessarily prove that they have all been absorbed by the leaves. If absorption of a nutrient A from a spray solution causes increased growth, the concentration in the plant of another nutrient B will tend to fall. This, and possibly an increase in the size of the root system, may lead to greater uptake of B from the soil. These causes of additional uptake of B could not increase the concentration of B above that in the controls, and therefore an increase in the content percentage of dry weight of any nutrient indicates that it has been absorbed from the spray. Expt. 8 showed that nitrogen, applied in the absence of phosphorus and potassium, was absorbed by leaves and increased plant growth. It is possible, therefore, that the increases in phosphorus and potassium contents obtained with the NPK sprays in other experiments were the result of uptake from the soil due to extra growth caused by the nitrogen in the spray. Small increases of N and K percentage of the dry matter, frequently in both roots and tops, were obtained in all experiments, but of phosphorus only in Expt. 6. There can be little doubt, therefore, that some potassium, as well as nitrogen, was absorbed from the mixed spray. The extent to which phosphorus and potassium may be absorbed by leaves can be determined only by using sprays supplying these nutrients singly.

Absorption of one nutrient by the leaves, as well as increasing the uptake of other nutrients from the soil, may also depress the absorption of the same nutrient by the roots. If this occurs the apparent absorption of this nutrient by the leaves, as measured by the difference in nutrient content between sprayed and control plants, will then be less than the real uptake. When the proportions of the three nutrients absorbed from sprays differed from their concentrations in the spray, some of the nutrients applied in the spray appeared not to have been absorbed. As there was never any sign of a salt deposit on the leaf surface, it is possible that all the nutrients in the spray had been absorbed but that uptake from the soil had been depressed. In the present experiments no estimate was made of the quantity of nutrients applied in the spray nor of the amounts washed off the leaves at harvest, so the fraction of the spray nutrients absorbed by the plant and the fraction deposited on the outside of the leaf were not known. Such measurements were made in experiments to be described in a subsequent paper. In order to demonstrate that uptake of a nutrient by the leaves depresses its uptake from the soil it is necessary to distinguish between the nutrients in the plant absorbed by the two routes. This would be possible if either the nutrient in the spray or in the supply to the roots were labelled, for example, with a radio-isotope.

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The Osmotic Inactivation *in situ* of Plant Mitochondrial Enzymes

GEORGE G. LATIES[1]

Kerckhoff Laboratories of Biology, California Institute of Technology

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SUMMARY

When cauliflower buds are gently disrupted in distilled water and homogenized immediately thereafter in hypertonic sucrose, the succinoxidase activity of particles prepared from such homogenates is considerably less than that of particles prepared from tissue homogenized in sucrose directly. Other enzymatic functions are impaired as well. When tissue is washed in water for any length of time prior to homogenization, the deleterious effect of hypotonicity is exaggerated. The irreversible injury to the mitochondria brought about by a hypotonic environment may be precluded to some extent by maintaining the temperature at 0° C. Washing injury is also minimized by washing tissue in hypertonic solutions.

In addition to the irreversible osmotic injury which the mitochondria may undergo, a reversible inhibition of succinic dehydrogenase may be effected by a soluble, heat-stable component of cauliflower juice. The latter inhibition appears to be competitive in nature.

The reduction of ferricyanide has been used as a means of measuring succinic dehydrogenase activity anaerobically. The concordance of manometric and potentiometric measurements of dehydrogenase activity suggests that the ferrocyanide ion may move freely from within the cell to the outer solution. There are indications as well that certain endogenous cell materials may be removed from the tissue during relatively short washing-periods.

INTRODUCTION

THE aerobic respiratory systems of certain higher plant tissues have recently been shown to be associated with intracellular particulates distinct from the plastids (Bonner and Millerd, 1953; Laties, 1953*a*). These findings have followed upon the excellent contributions from many sources (Harman, 1950*a*; Hogeboom, 1951) linking the oxidative respiratory enzymes of animal tissues to the mitochondria. Although it is now possible to obtain many of the respiratory enzymes *in vitro* by careful separation of the mitochondria from homogenates of plant tissues (Laties, 1953*b*), early attempts to study these self-same enzymes in homogenates, without further fractionation, were relatively unsuccessful. In particular, some difficulty was experienced in demonstrating the succinoxidase system (Bonner and Wildman, 1946), which system was considered, on the basis of indirect evidence, to be operative in the intact tissue.

Studies undertaken to investigate this anomaly have produced at least two explanations for the difficulties encountered with homogenates. The

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[1] Present address: Botany Department, University of Michigan.

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non-specific and irreversible deleterious effects of certain soluble components of the protoplast of cauliflower bud cells upon the oxidative mitochondrial enzymes, including succinoxidase, were shown to be dependent upon the tonicity of the environment, injury resulting primarily under hypotonic conditions (Laties, 1953*b*). In addition, cauliflower bud homogenates contain a competitive inhibitor of succinic dehydrogenase which will be described below. When cauliflower buds are gently disrupted with mortar and pestle the respiration of the intact pieces is shown to be affected by the competitive inhibitor in the ambient juice. Experiments in which the whole tissue pieces surviving light grinding were washed to remove the juice led to the observation that the respiratory activity of the mitochondria subsequently isolated from this tissue was profoundly affected by the initial washing of the whole tissue. This observation and its implications will also be considered herein.

At the outset of this investigation it was deemed desirable to have a way of estimating the functional concentration *in vivo* of succinic dehydrogenase, at least, in order to be able to relate the activity of the homogenate to the activity of the intact tissue. To this end the manometric method of measuring dehydrogenase activity described by Quastel and Wheatley (1938) was used, in which ferricyanide acts as electron acceptor, while CO_2 is released into the gas phase from bicarbonate buffer as a consequence of the formation of hydrogen ion during the reduction of ferricyanide. Dehydrogenase measurements upon whole tissue with this method proved sensitive and reproducible. The extent to which the method may be considered specific for a given dehydrogenase will be discussed.

Recently Hochster and Quastel (1952) have described the experimental substitution of MnO_2 for O_2 as the final respiratory hydrogen acceptor. When the respiratory activity of intact tissue is measured anaerobically with MnO_2 as the terminal hydrogen acceptor, catalytic quantities of ferricyanide must be used to link the cellular oxidative systems with MnO_2 which, because of its insolubility in water, fails to penetrate the tissue. The possibility of the utilization of this artificial respiratory system in the analysis of the precise relation of respiration to certain physiological phenomena, such as growth and salt accumulation, makes further information pertaining to the utilization of ferricyanide in studies of plant-tissue respiration of general interest.

METHODS

Heads of cauliflower (*Brassica oleracea*) were obtained in local markets. The heads were washed in tap-water, rinsed in distilled water, and blotted dry. The topmost 2–3 mm. of immature inflorescence, designated below as cauliflower buds, was removed either with a scalpel or plastic grater, and used as follows:

In experiments involving studies of whole tissue pieces a quantity of buds, usually 5–6 g., was ground in a mortar with 4 volumes of water or designated solution. This grinding, done gently in the absence of sand, was designed primarily to reduce the bud pieces to a size range such that a suspension could be pipetted with a pipette from which the tip had been removed. Subsequently, comparable tissue samples were secured by weighing aliquots of the rinsed and blotted residue obtained by filtering the suspension through cheesecloth. Such samples were added directly to manometer vessels, the tissue being kept out of the centre-well by temporarily inserting a glass plug in the latter. Approximately 50 per cent. of the initial bud material normally remained in the residue following light grinding and filtration. Particles were prepared as described elsewhere (Laties, 1953*a*, *b*). In early experiments homogenization was carried out in water or buffer, using one part of tissue, one-third part of sand, and 6–7 parts solution. All solutions, together with mortar and pestle, were kept cold. In later experiments homogenization was performed with ice-cold 0·5 M. sucrose. The manner in which homogenization was carried out is specified in the legends of the pertinent tables.

The anaerobic measurements of succinic dehydrogenase were carried out manometrically in the manner described by Quastel and Wheatley (1938). This method depends upon the formation of hydrogen ions as a consequence of the reduction of ferricyanide by substrate hydrogen atoms. Hydrogen ions so formed release CO_2 from bicarbonate buffer with a consequent increase in pressure. For each mole of succinate oxidized two moles of CO_2 are released. In practice the vessels are first gassed for 2–3 minutes with a mixture of 5 per cent. CO_2 in nitrogen. Following a 10-minute equilibration period during which the taps are occasionally opened to release the pressure, a mixture of ferricyanide and bicarbonate is introduced from the sidearm into the main chamber, wherein tissue and various reagents are contained in $2{\cdot}5\times10^{-2}$ M. bicarbonate buffer. Alternatively the ferricyanide mixture may be added following measurement of the endogenous activity in the absence of ferricyanide. A stick of yellow phosphorus in the centre well ensures the removal of traces of oxygen. Succinoxidase measurements were carried out manometrically in an air atmosphere, using the direct method of Warburg (Umbreit *et al.*, 1949).

The washing of tissue pieces was carried out in one of several ways. Following the light grinding procedure, and subsequent filtration of the suspension through cheesecloth, the residue was placed in a litre beaker and washed by stirring with a power-driven glass stirrer. The tissue was in this manner submitted to from 3 to 6 consecutive washings of 20-minute duration, being filtered and resuspended after each washing. Alternatively the residue was enclosed in a piece of cheesecloth, the cheesecloth then being fashioned into a bag and attached to the glass stirrer. The tissue was then swirled gently either in continuously running tap-water or in changes of water or solution, as specified.

RESULTS

The relation of CO_2 evolution to succinic dehydrogenase activity

When the evolution of CO_2 from a reaction mixture containing bicarbonate buffer and ferricyanide is to be taken as a measure of the H^+ production related to ferricyanide reduction, it must first be established that neither extraneous acid formation nor metabolically generated CO_2 is responsible for any significant fraction of the evolved gas. To distinguish whether CO_2 arises in the tissue *per se* or whether the observed CO_2 is released from the buffer solution as a consequence of H^- production, the quantity of CO_2 evolved may be compared to the quantity of bicarbonate disappearing. The bicarbonate concentration at any time is estimated from a measure of the increase in pressure effected when acid is introduced into the main chamber from the sidearm. Thus acid is added to one of two replicate reaction mixtures at the beginning, and to the other at the end, of the experimental period. The bicarbonate disappearance is calculated from the difference in pressure increments arising from the addition of acid, and may be compared to the quantity of CO_2 evolved during the experimental period.

Table I presents typical experimental data pertaining to measurements obtained with water-washed tissue. Although the activity in the absence of substrate decreases sharply with time, the activity attributed to succinic dehydrogenase remains reasonably constant. The origin of the evolved CO_2 in the absence of substrate has been analysed by the above method. In the presence of ferricyanide, approximately half the control value is due to displacement of CO_2 from bicarbonate, while half originates as CO_2 as such. Consistent with this observation is the finding that in the absence of ferricyanide the evolution of CO_2 is approximately half that of the control in the presence of ferricyanide, and the origin of CO_2 in this case is almost entirely from endogenous decarboxylations. Succinate is without effect in the absence of ferricyanide.

TABLE I

Succinic dehydrogenase activity of cauliflower bud pieces

Time in minutes	μl.CO_2/400 mg. fresh weight/10 minutes		
	Control	+ Succinate	Succinic dehydrogenase activity
5	23	22	—
10*	23	22	—
15	18	31	—
25	30	90	60
35	15	74	59
45	18	73	55
55	11	64	53

* Ferricyanide added after 10 minutes. Final concentration of reagents in manometer flasks as follows: $NaHCO_3$, 2·5 · 10^{-2} M.; $K_3Fe(CN)$, 1·2 · 10^{-2} M.; succinate, 4 · 10^{-2} M. (brought to pH 7·2 with KOH); 400 mg. water-washed tissue per flask. Final volume brought to 3·0 ml. with glass-distilled H_2O. Gas phase, 5% CO_2 in nitrogen.

In order to keep the pressure changes following the addition of acid within the range of the manometer, the bicarbonate concentration must be reduced to approximately one-fourth that normally employed. This reduction of concentration presents no difficulty in the absence of substrate, but in the presence of 4×10^{-2} M. succinate the molar ratio of succinate to bicarbonate is approximately 6·6, and the H^+ binding capacity of succinate is no longer inconsequential, compared to that of bicarbonate. Under these conditions there is no longer a direct relation between the reduction of ferricyanide and the release of CO_2 from bicarbonate, and an analysis of the origin of CO_2 has no quantitative significance. A discrepancy of from 22 to 25 per cent. of the expected CO_2 evolution was calculated when 4×10^{-3} m. eq. of H^+ was added from the sidearm to 2×10^{-2} m. eq. of bicarbonate in the presence of 0·04 M. succinate.

To determine whether any part of the CO_2 released in response to the addition of succinate was due to the direct formation of CO_2 by the tissue, the evolution of CO_2 was determined from tissue maintained in phosphate buffer containing the usual concentration of ferricyanide. In such experiments pure nitrogen or argon was substituted for a nitrogen-carbon dioxide mixture in the gas phase. The carbon dioxide formed during the experimental period was calculated from the pressure readings observed in replicate reaction mixtures in which acid was introduced from the sidearm at the beginning and end of the experiment respectively (see Umbreit *et al.*, 1949). The CO_2 arising as a consequence of succinate addition was compared to the succinate-induced CO_2 release observed in concomitant experiments carried out in the regular manner in bicarbonate buffer. The relative quantities of CO_2 arising in phosphate and bicarbonate buffer in a 50-minute period were 8 μl. and 195 μl. respectively. The evolution of CO_2 in solutions of ferricyanide and bicarbonate in response to succinate addition may thus be attributed almost exclusively to H^+ production.

The method described above makes no distinction between H^+ arising as a consequence of ferricyanide reduction by dehydrogenase activity and H^+ arising from direct metabolic acid formation. The reduction of ferricyanide was therefore measured directly by potentiometric measurements of the oxidation-reduction potential of a ferricyanide-ferrocyanide mixture. Potentiometric measurements were carried out upon the same reaction mixtures employed for the manometric determination of dehydrogenase activity. The ratio of ferri- to ferrocyanide was initially 3 in these experiments. A standard curve was prepared describing the potential of an inert gold electrode with reference to a standard calomel half-cell, as a function of the ratio of ferri- to ferrocyanide. The extent of ferricyanide reduction during the experimental period was calculated from this curve, the experimental period being adjusted so as to keep the potential measurements within the well-poised, relatively straight portion of the curve. At the concentration of salts used, the ferri-ferrocyanide system determines the electrode potential to the exclusion of any other system, whether or not tissue and substrate is present (Fig. 1). Thus the potential being measured is truly the oxidation-reduction potential of the ferri- ferrocyanide system (Michaelis, 1930).

At the beginning of each experiment, 0·2 ml. 5N H_2SO_4 was tipped into one of two replicates of each experimental treatment. This addition was followed by 1 ml. 0·5 M. phosphate buffer (pH 7·0) and 0·2 ml. 5N NaOH. The reaction mixture so treated was placed in the constant-temperature bath along with the replicate in which CO_2 evolution was measured. At the end of the experimental period acid was added to the second vessel of each pair, followed by buffer and alkali. The contents of each manometer flask were then transferred to 10-ml. specimen tubes, the volume brought to 5·0 ml., and the oxidation-reduction potential of the ferri- ferrocyanide system measured, using the gold electrode and a sensitive potentiometer. From the data in Table II it is clear that the CO_2 taken as a measure of succinic dehydrogenase activity is due entirely to the reduction of ferricyanide and originates neither as CO_2 nor as a consequence of extraneous acid formation.

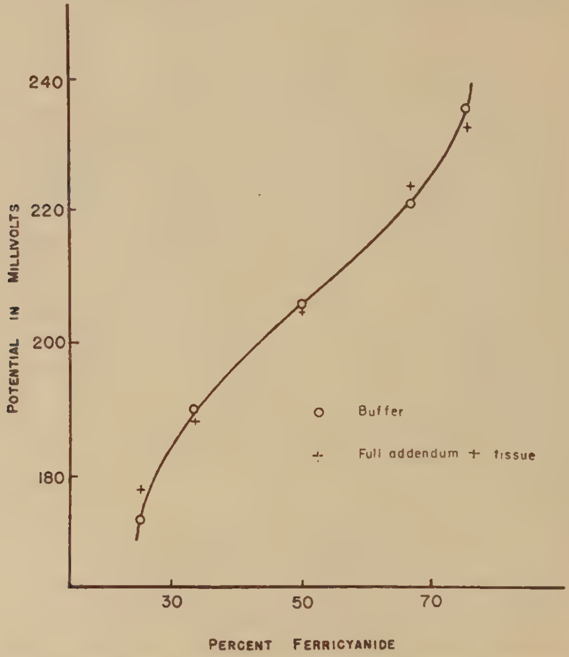

Fig. 1. The effect of tissue and substrate additions on the oxidation—reduction potential of ferricyanide—ferrocyanide mixtures. Final concentration of reagents as follows: ferri-ferrocyanide, total concentration, 6×10^{-3} M.; phosphate buffer, pH 7·1, 10^{-1} M. In addition, where full addendum is indicated: succinate, $2·5\times10^{-2}$ M.; bicarbonate, $1·3\times10^{-2}$ M.; 400 mg. washed tissue.

The question which remains is whether the reduction of ferricyanide is attributable entirely to succinic dehydrogenase activity. In this regard, when fumarate or malate were given as substrates, no evolution of CO_2 over that of the controls was observed, indicating that the CO_2 evolution elicited by the addition of succinate represented a measure of the succinic dehydrogenase activity of the tissue. It does not follow that this method is unsuitable for the assay of other dehydrogenases. Although cauliflower-bud tissues are known to contain enzyme systems capable of carrying out the entire tricarboxylic acid cycle (Laties, 1953*a*, *b*), it will be shown below that extensive washing of whole tissue destroys the activity of many mitochondrial enzymes, and it has also been demonstrated (Laties, 1953*b*) that the malic oxidase system is particularly subject to such inactivation.

TABLE II

Comparison of manometric and potentiometric determinations of succinic dehydrogenase activity

Final concentration of reagents in manometer flasks as follows: $NaHCO_3$, $2{\cdot}5 \times 10^{-2}$ M.; $K_3Fe(CN)_6$, $3{\cdot}8 \times 10^{-3}$ M.; $K_4Fe(CN)_6$, $1{\cdot}2 \times 10^{-3}$ M.; succinate $4{\cdot}0 \times 10^{-2}$ M. 400 mg. tissue per flask, or the equivalent of this quantity as water or filtrate homogenate. Final volume 4·0 ml. Filtrate obtained by lightly grinding buds with 4 parts glass-distilled H_2O and filtering through cheesecloth. Homogenate prepared by grinding water-washed tissue in 6 parts ice-cold glass-distilled H_2O or filtrate in a chilled mortar with sand. Reduced ferricyanide concentration designed to permit large fractional change in ferricyanide concentration for potentiometric measurements.

Experiment and duration	Material and treatment	μ moles CO^2 evolved (measured manometrically)			μ moles $K_3Fe(CN)_6$ reduced (measured potentiometrically)
		Control	+ Succinate	Diff.	
1 45 minutes	Water-washed suspension	113	242	129	126
	As above, plus filtrate	160	165	5	0
2 60 minutes	Water-washed suspension	98	388	290	300
	As above, high bicarbonate ($7{\cdot}5 \times 10^{-2}$ M.)	98	218	120	127
3 45 minutes	Water-washed suspension	112	224	112	121
	As above, homogenized	95	200	105	110
	Homogenate plus filtrate	177	222	45	29

The inhibition of succinic dehydrogenase by cauliflower juice

When the succinic dehydrogenase activity of cauliflower bud minces was measured as a function of tissue concentration, a lack of proportionality was observed which in extreme cases indicated little or no succinic dehydrogenase activity at high tissue concentrations. This anomalous behaviour suggested the presence of an inhibitor in the suspension, which supposition was borne out by several types of experiment. Tissue pieces were separated from the crude mince by filtration of the latter through cheesecloth. The succinic dehydrogenase activity of such pieces, rinsed in buffer or distilled water, was found to be proportional to tissue concentration. When washed pieces were resuspended in filtrate, inhibition was again manifest (Table III). That such inhibition was not an artifact arising from the H^+ binding capacity of the filtrate was indicated by the inhibitory effect of the filtrate upon ferricyanide reduction as determined by direct potentiometric measurements (Table II).

TABLE III

The inhibition of cauliflower bud succinic dehydrogenase by an endogenous inhibitor

Contents of manometer flasks as follows: $NaHCO_3$, $2{\cdot}5 \times 10^{-2}$M; $K_3Fe(CN)_6$, $2{\cdot}5 \times 10^{-2}$ M.; succinate, 4×10^{-2} M.; final volume 3·0 ml. for tissue suspension, and 4·0 ml. for homogenates and particle suspensions. Filtrate prepared by lightly grinding buds in 4 parts of water and filtering suspension through cheesecloth. Where KCN treatment is indicated, filtrate made 0·01 M. with respect to KCN (adjusted to pH 7·0) and allowed to stand several hours, together with the appropriate control.

			μl.CO_2/400 mg. fresh wt./10 minutes		
Expt.	Experimental material	Addendum	Control	+ Succinate	Succinic dehydrogenase activity
1	Water-washed suspension	None	33	83	50
		Filtrate	45	43	0
2	Water-washed suspension	None	20	55	35
		Filtrate	31	34	3
3	Water-washed suspension	None	26	94	68
		Boiled filtrate	34	52	18
		Boiled filtrate, dialysed	23	82	59
		Boiled filtrate, KCN treated	28	75	47
4	Water homogenate of washed tissue	None	16	42	26
		Filtrate	23	35	12
5	Particulate preparation from washed tissue, prepared in water	None	7	61	54
		Filtrate	7	46	39
6	As above	None	6	44	38
		Filtrate	11	30	19

Several observations which followed upon studies pertaining to the effect of the filtrate upon the succinoxidase activity in air led to the assumption that the inhibition described above was competitive in nature. Tissue pieces separated from a mince and washed in water showed no respiratory increment in terms of oxygen consumption upon the addition of succinate. In the presence of filtrate, however, a response to succinate was manifest, in distinct contrast to the observed response to filtrate under anaerobic conditions. Filtrate alone was shown to have negligible succinic dehydrogenase and succinoxidase activity. The increase in ferricyanide reduction in the presence of filtrate, evident in both the control and experimental reaction mixtures, was attributable to non-enzymic reduction, since this increase was also effected by boiled filtrate. The apparent response to succinate demonstrable in the presence of filtrate in aerobic experiments appeared to be a consequence of the diminution of the control rate of respiration by filtrate. The maximum respiratory rate obtainable with or without filtrate or succinate was the same. The effect of filtrate addition could be simulated by malonate, a response to succinate being evident only after partial inhibition of the endogenous respiration. Under anaerobic conditions, in the presence of ferricyanide, malonate was found to exert no inhibition whatsoever on the endogenous activity of the controls, but was found to inhibit completely the increment obtained by the addition of succinate.

Numerous respiratory studies with tissues of higher plants have indicated that many, if not all, of the respiratory dehydrogenase enzymes are substrate saturated in intact tissue (Bonner and Wildman, 1946; Bonner, 1948). At least, the addition of exogenous substrate elicits no respiratory response. In distinction to certain animal tissues and many microbial cells, the tissues of higher plants are not readily depleted of endogenous substrate by relatively short incubation periods. It would appear that in the experiments cited above the succinic dehydrogenase system is substrate saturated under aerobic conditions but that such is not the case anaerobically. Although the failure to produce succinate endogenously under anaerobic conditions may be ascribed to the inability of certain dehydrogenases to react with ferricyanide, an alternative explanation would suggest that the rate of reaction with ferricyanide is simply too slow in washed tissue to result in the production of appreciable quantities of succinate. Ferricyanide has been shown to be a suitable electron acceptor for the substrate oxidations of the tricarboxylic acid cycle (Cross *et al.*, 1949).

If, in fact, the endogenous production of succinate is minimized anaerobically, an explanation is at hand for the ability of filtrate to cause complete inhibition of succinic dehydrogenase anaerobically where only partial inhibition of the same activity is obtained under aerobic conditions. Such an explanation assumes the inhibition to be competitive, and therefore to be a function of the ratio of inhibitor to substrate. The inhibition by the filtrate of the succinic dehydrogenase activity of homogenates or isolated particles has been shown to be consistently less than the inhibition of the succinic dehydrogenase activity of whole tissue pieces (Table III). The simplest interpretation of this observation consistent with the above hypothesis would suggest that the ratio of inhibitor to substrate is lower where there are no penetration barriers into the cell.

A more rigorous proof of the competitive nature of the inhibitor was carried out by a kinetic analysis such as described by Lineweaver and Burk (see

Wilson, 1950). As will be indicated below, and as has been described elsewhere (Laties, 1953*b*), there are components of cauliflower juice which effect irreversible inhibition of the succinic dehydrogenase system as well as of other enzyme systems. Irreversible injury by juice components may be largely precluded by the grinding or homogenization of tissue in ice-cold hypertonic solution instead of in water or dilute buffer. To distinguish clearly between the competitive and non-competitive inhibitors in cauliflower juice a comparison was made between the inhibition resulting from the preparation of particles in distilled water, and the inhibition resulting from the presence of juice under hypertonic conditions during the experimental period. Particles were prepared both in ice-cold 0·5 M. sucrose and in distilled water. The succinic dehydrogenase activity of particles prepared in both ways was then measured as a function of substrate concentration, the particles prepared in sucrose being tested both in the presence and absence of separately prepared boiled filtrate. In Fig. 2 the reciprocal of the rate of the succinic dehydrogenase activity is related to the reciprocal of the substrate concentration.

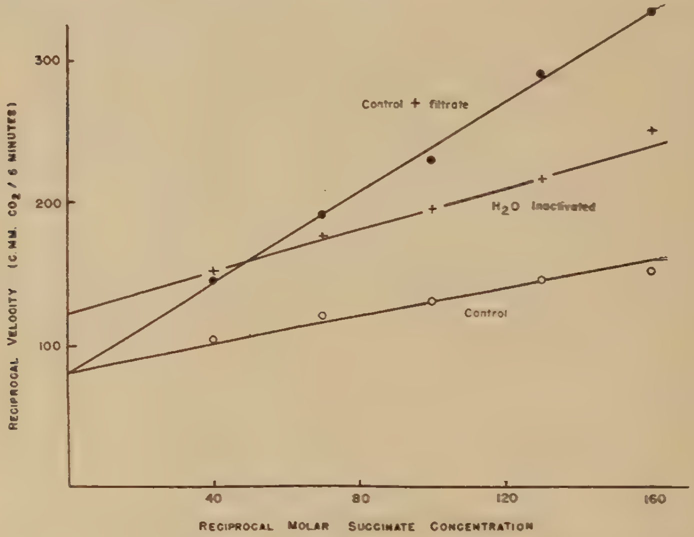

FIG. 2. Analysis of inhibition of cauliflower bed succinic dehydrogenase. (See text.)

When the straight lines describing this relationship transect the same point on the ordinate axis it may be considered that a competitive inhibitor is present in the reaction mixture having the lower activity at a given substrate concentration. When the line describing the reciprocal of the activity of an inhibited sample as a function of the reciprocal of the substrate concentration has a steeper slope than that for the uninhibited sample, and when this line passes through a different point on the ordinate axis, the inhibition is considered to be non-competitive (Wilson, 1950). Thus the inhibition effected by preparation of the particles in water is clearly distinct from that due to the presence of the filtrate during the experimental period, the latter inhibition being of a competitive type. As will be discussed later, there remains the possibility that substrate simply protects the enzyme from inactivation by a component of the filtrate, the extent of inhibition depending upon the ratio of inhibitor to substrate (Ames and Elvehjem, 1942). If this should be the case, it is clear from Fig. 3 that such inactivation is reversible, since, when the tissue is removed from the filtrate, the initial activity is re-established.

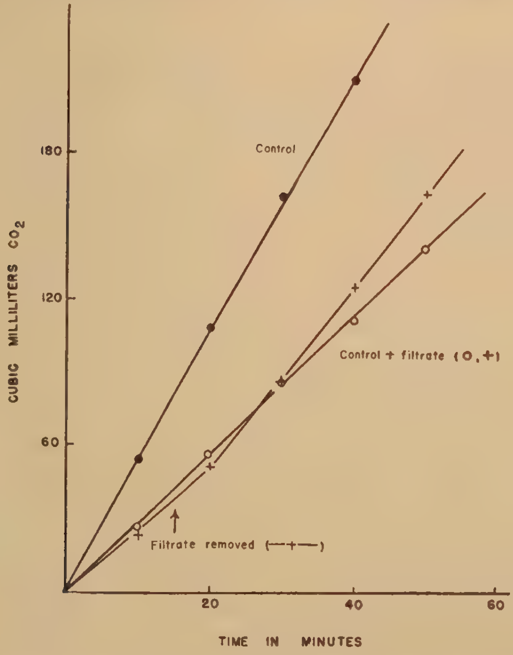

FIG. 3. The reversible nature of succinic dehydrogenase inhibition by endogenous inhibitor. Final concentration of reagents as in Table I. Filtrate prepared by grinding buds with one part of water, boiling for 5 min., and filtering through muslin. 1·5 ml. filtrate per flask. Where designated, manometer cup contents were passed through muslin after 15 min., the tissue rinsed, and resuspended in the absence of filtrate.

Although the precise nature of the competitive inhibitor remains to be elucidated, certain characteristics have been examined. The inhibitor is heat-stable and dialysable. The combination of inhibitor with cyanide results in an inactive complex (Fig. 4). Of the several compounds which might be expected to inhibit succinic dehydrogenase competitively [malonate (Quastel and Wheatley, 1931), pyrophosphate (Leloir and Dixon, 1937), oxalacetate (Swingle *et al.*, 1942)], only oxalacetate would be expected to combine with cyanide. On the other hand, it does not seem likely that oxalacetate would survive the boiling procedure to any appreciable extent. However, many sulphydryl enzymes such as succinic dehydrogenase are inhibited by dithio compounds (Ames and Elvehjem, 1942), and such dithio compounds are cleaved by HCN. As will be indicated in the discussion, inhibition by dithio compounds may simulate a competitive inhibition.

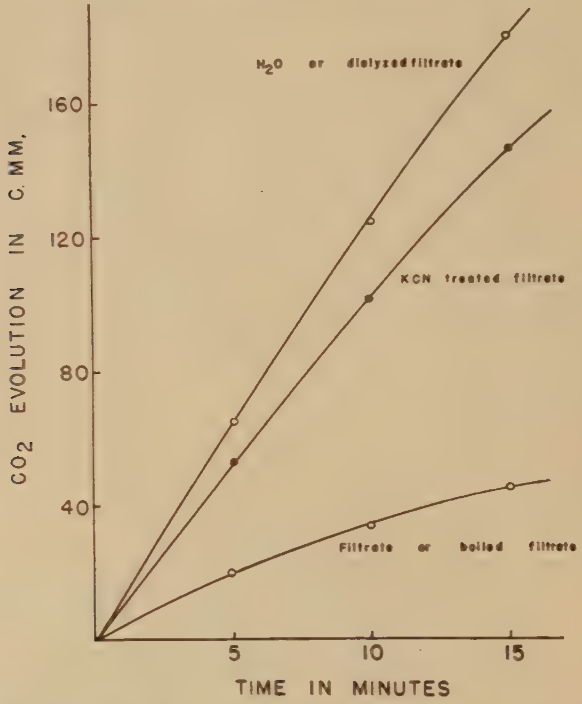

FIG. 4. The effect of cyanide on the inhibitory characteristics of the endogenous succinic dehydrogenase inhibitor. Contents of manometer flasks as in Table I. Filtrate added where indicated. Cyanide-treated filtrate made 0·01 M. with KCN, pH 7·0. After 4 hrs. incubation period, excess cyanide removed by aeration prior to experiment.

The effect of tissue washing on the activity of particulate enzymes

When it was recognized that cauliflower juice contained an effective competitive inhibitor of succinic dehydrogenase, a procedure was adopted in which tissue pieces were washed following the initial light grinding to rid the pieces of juice components. This washing was intended to simulate the washing technique employed by Keilin and Hartree (1947), which raised the specific activity of the succinoxidase of heart-muscle particulates. The experiments reported in the first section herein were all carried out with tissue washed from 1 to 2 hours in changes of cold water.

It is not clear whether the washing procedure actually removes inhibitor from the cells or whether washing simply removes contaminating solution adhering to the tissue pieces. It has been observed that recovery of the tissue succinic dehydrogenase activity in homogenates is almost completely achieved when washed tissue is homogenized in water (Expts. 3 and 4, Table IV), but is very poor when tissue is homogenized in the initial solution resulting from light grinding. Such a distinction suggests that inhibitor may actually be removed from the tissue.

Whether or not inhibitor is washed from the cells, it appears that other cytoplasmic components may diffuse into the bathing medium under certain conditions. For example, when tissue pieces are bathed in 5×10^{-2} M. bicarbonate instead of water, the endogenous release of carbon dioxide in the absence of ferricyanide is greatly diminished (Expts. 1 and 2, Table IV). Unless it is assumed that the external pH affects intracellular decarboxylating systems, the most reasonable interpretation would seem to be that either requisite substrate or cofactors are removed from the tissue by alkaline washing. Some support is given this view by studies of the effect of tissue homogenization upon the endogenous blank. When tissue pieces are homogenized there appears to be little change in the endogenous blank. However, if the homogenate is cleared of debris by low-speed centrifugation the greater part of the succinic dehydrogenase activity of the initial homogenate remains in the supernatant, whereas the endogenous blank is sharply reduced (Expt. 6, Table IV). The endogenous blank is thus largely, although not exclusively, confined to the intact tissue. To explain the relative constancy of the blank following homogenization it would have to be assumed that substrates or cofactors supplied by the disrupted cells raise the endogenous activity of the surviving tissue pieces. With respect to the disrupted cells, however, the substrate level may be thought to be diminished by dilution. The variable effectiveness of the washing procedure would then explain the different extent to which the endogenous blank is manifest in homogenates cleared of whole cells (Table IV, Expt. 5 compared to Expt. 6).

When particles are isolated by high-speed centrifugation from a water homogenate, both the blank and the control activity in the presence of ferricyanide are almost completely abolished. The enzyme systems, substrates, or both, responsible for the blank and control activity are thus primarily in the non-particulate fraction of the cytoplasm. When the activity of the control is diminished, either naturally during the course of the experiment (Table I) or by alkaline washing of the tissue (Table IV), the decrement in absolute terms is in each case of the order of magnitude of the decrement of the blank, and is probably to be attributed to the decrease in the blank.

To reduce the blank as indicated in Table IV, bicarbonate washing must be carried out for some time. In contrast, however, there is an immediate effect of bicarbonate on the level of competitive inhibitor in solution following the initial light grinding. As may be seen in Table IV, Expts. 7, 8, relatively little inhibition is exerted by the ambient solution when grinding has been carried out in 5×10^{-2} M. bicarbonate (final pH approximately 8·0). The pH may conceivably affect either the formation of inhibitor or its penetration into the tissue. Since the addition of bicarbonate following the grinding procedure does not relieve the inhibition, the first alternative appears most likely.

TABLE IV

The effect of tissue treatment on the endogenous blank

Contents of manometer flasks as in Table III. Blank represents activity without $K_3Fe(CN)_6$ and without succinate. Control represents endogenous activity with $K_3Fe(CN)_6$. The blank and control are not to be compared on an absolute basis since the values presented are sequential; i.e. the blank was observed for 10–15 minutes, following which ferricyanide was added and the control values obtained thereafter. Table I indicates the blank diminishes with time. In Expts. 7 and 8, tissue transferred to manometer flasks in the medium in which tissue was ground. Washing carried out for 1–2 hrs. where indicated.

		μl.CO_2/400 mg. fresh wt./10 minutes			
Expt.	Experimental material and treatment	Blank	Control	+ Succinate	Succinic dehydrogenase activity
1	Water-washed suspension	23	26	77	51
	Bicarbonate-washed suspension	12	13	63	50
2	Water-washed suspension	39	37	60	23
	Bicarbonate-washed suspension	0	15	35	20
3	Water-washed suspension	29	26	61	35
	As above, homogenized	18	17	45	28
4	Water-washed suspension	36	32	61	29
	As above, homogenized	40	29	61	31
5	Homogenate of washed tissue	30	26	53	27
	Supernatant following centrifugation ($500 \times g$)	1	9	31	22
	Filtrate following filtration through cheesecloth	2	10	29	19
6	Homogenate of washed tissue centrifuged ($500 \times g$)	21	19	55	36
	As above, centrifuged ($3{,}000 \times g$) supernatant	18	14	31	17
	Resuspended particles	0	4	25	21
7	Suspension of unwashed tissue ground in H_2O	30	25	34	9
	Ground in bicarbonate	31	22	48	26
8	Suspension of unwashed tissue				
	Ground in H_2O	26	17	26	9
	As above, filtered	12	9	26	17
	Ground in bicarbonate	21	18	34	16

When experiments were undertaken to study the enzymatic characteristics of cell particles there appeared no further need for the light grinding and washing procedure since the centrifugal separation of the particles from the soluble components of the homogenate seemed an adequate means of removing the inhibitor. When the succinoxidase activity of cellular particles was studied it became apparent that the activity of particles prepared from unwashed tissue was considerably greater than that of particles from washed tissue. The same proved true of both the succinic dehydrogenase and cytochrome oxidase activities, although to a considerably lesser extent, so that the effect of washing upon the succinoxidase system could not be entirely attributed to the effects upon the succinic dehydrogenase and cytochrome oxidase systems. Thus, in experiments with whole tissue, washing appeared advantageous by dint of the removal of the inhibitor. However, in particle suspensions from which inhibitor was removed during the preparation, the deleterious effects of the previous tissue washing became manifest.

Washing injury was shown to have an appreciable temperature coefficient, to be independent of the presence of oxygen, and to be a function of the tonicity of the environment (Table V, Expts. 1–4). Hypertonic mannitol or KCl solutions were similar to sucrose solutions in their ability to lessen the deleterious effects of washing. The data of Expts. 1–4, Table V, indicate that washing *per se* is not the cause of the observed inactivation, but rather that inactivation is a function of the tonicity of the environment and the temperature. Although the effect of hypotonic treatment is exaggerated with time (Expt. 5, Table V), a considerable response to the tonicity of the environment is observed in the short period required to carry out the light grinding (Expt. 6, Table V). The temperature-sensitive destruction of certain mitochondrial enzymes following the exposure of isolated mitochondria to soluble cauliflower juice components in a hypotonic environment has been described (Laties, 1953*b*). The injury occurring to certain respiratory enzymes when whole tissue pieces are submitted to a hypotonic environment appears to be of the same kind.

The question arises as to whether the injury occurring immediately upon grinding is a consequence of the presence of soluble components of the protoplast released during the light grinding. In Expt. 7, Table V, the tissue was first ground lightly in ice-cold 0·5 M. sucrose following which the tissue pieces were filtered from the suspension, rinsed in cold sucrose, and then subjected to distilled water or bud filtrate for 2–3 minutes at room temperature. The data indicate that mere exposure to distilled water is sufficient to effect injury. Although the preparation of particles in ice-cold 0·5 M. sucrose has proved the most satisfactory manner of obtaining particles high in succinoxidase, malic oxidase, and α-ketoglutaric oxidase activity, it is clear that considerable diminution of both tissue and particle activity may occur when either tissue pieces or particle suspensions are incubated in 0·5 M. sucrose at room temperature. The protection to certain respiratory enzymes afforded by incubation or washing of tissue in 0·5 M. sucrose is relative. The lower activity of particles from tissue bathed in 0·5 M. sucrose, at room temperature, compared to that of particles prepared from tissue directly, in the cold, suggests that any disorganization of the endogenous osmotic relationships may, at higher temperature, result in partial inactivation of certain mitochondrial enzymes. Consistent with this suggestion is the work of Opie (1948) wherein morphological changes of liver mitochondria are described following the introduction of liver slices into hypertonic or isotonic solutions as well as into hypotonic solution.

TABLE V

The effect of whole tissue washing on the activity of cellular particulates

Final concentration of reagents in manometer flasks as follows:

In Expts. 1–4, KH_2PO_4–Na_2HPO_4 (pH 7·2), $3·3 \times 10^{-2}$ M.; cytochrome C, 2×10^{-5} M.; succinate 5×10^{-2} M.; 0·5 ml. enzyme, final volume 3·0 ml. 0·2 ml. 10% KOH in the centre well together with cm.-square fluted filter-paper. Initial light grinding in H_2O, subsequent washing, 1 hr.; final homogenization in ice-cold H_2O. Control represents tissue homogenized directly in ice-cold H_2O without light grinding or washing.

In Expts. 5–7, 0·6 ml. 1·5 M. sucrose added to manometer flasks in addition to other reagents to maintain high osmotic pressure (see Laties, 1953 *b*). Control represents tissue homogenized directly in ice-cold 0·5 M. sucrose.

Expt.	Treatment	Succinoxidase activity of particles $Q_{O_2}(N)$
1	Control	242
	Washed in H_2O	133
	Washed in 0·5 M. sucrose	220
2	Control	288
	Washed in H_2O	159
	Washed in H_2O, 2° C.	260
3	Control	262
	Washed in H_2O	167
	Washed in H_2O, argon atmosphere	179
4	Control	319
	Ground in H_2O, incubated in minimal H_2O volume	194
	Ground in H_2O, filtered, incubated in moist atmosphere	177
	As above, incubated at 2° C.	232
	Ground in 0·5 M. sucrose, filtered, incubated in moist atmosphere	306
5	Control	300
	Homogenized directly in H_2O	203
	Ground in H_2O, washed in H_2O	118
	Ground in sucrose, washed in sucrose	180
6	Ground in H_2O, immediately rinsed in sucrose, homogenized at once	183
	Ground in sucrose, then treated as above	285
7	Control	224
	Ground in sucrose, rinsed in sucrose (2° C.)	287
	Ground in sucrose (2° C.), rinsed in H_2O (room temp.)	94
	Ground in H_2O, rinsed in sucrose (room temp.)	144

DISCUSSION

Current investigations of the respiratory metabolism of higher plant tissues have indicated that the enzymes involved are largely associated with discrete intracellular particles (Bonner and Millerd, 1953; Laties, 1953*a*, *b*). Although in the higher plants it has not been established rigorously that the biochemical entity, the cyclophorase system, is exclusively associated with the morphological entity, the mitochondrion, the size and staining properties of the particles comprising an active preparation suggest that the particles are primarily mitochondria. There is little doubt of the identity of comparable particles in preparations from animal tissues (Harman, 1950*a*). Neither chloroplasts nor developed leucoplasts are present in cauliflower florets,[1] with the consequence that there is less ambiguity regarding the origin of particles derived from homogenized cauliflower buds than there might be with respect to particles from homogenized mature leaves, for example.

The osmotic relationships of mitochondria within intact tissue

Isolated mitochondria are morphologically altered by exposure to hypotonic solution (Harman, 1950*b*), and with this alteration there is a concomitant loss of enzymatic activity (Harman, 1950*b*; Hogeboom, 1951). The work reported above suggests that the mitochondria, while *in situ*, suffer fundamental transformations as a consequence of exposure of tissue pieces to an environment of low osmotic pressure. Cytological observations of such changes are not new, but little attention has been given to the metabolic implications of such changes occurring within intact plant tissues. Opie (1948) has comprehensively described the effects upon liver mitochondria of exposure of liver slices to water. Zollinger (1948) has studied similar systems with the aid of the phase microscope. In both studies, exposure of tissue slices to water resulted in an almost immediate swelling and vesiculation of the mitochondria. Opie (1948) describes in addition the loss of basic staining properties of the intracellular particles in the presence of water. He points out, furthermore, that almost any osmotic change from the uncharacterized intracellular environment of the particles results in a visible alteration of the mitochondria. The biochemical consequences of the morphological alterations were adumbrated by Elliott and Libet (1942) in a study wherein brain minces prepared in water respired to a considerably lesser extent than minces similarly prepared in isotonic medium. Since minces essentially represent coarsely divided tissue pieces, it follows that injury to the respiratory systems was done *in situ*.

Buvat (1953) has described the way in which the mitochondria of certain plant cells undergo changes *in situ* following exposure of tissue pieces to water. Relatively soon after exposure cyclosis was observed to stop, and the mitochondria to swell and aline themselves end-to-end in heterogeneous string-like bodies. This phenomenon was largely temperature-dependent, being essentially precluded at 0° C. (Buvat, 1947). Similar temperature relationships were reported by Opie with respect to morphological changes, and were also observed in the experiments reported above, with respect to enzymatic changes. Buvat (1953), however, describes the remarkable situation wherein after some 3 days of exposure of the tissue to continually changed hypotonic medium, the cells are observed to 'adapt' to the environment. That is, cyclosis is resumed, the string-like aggregations of mitochondria break up, and the cell contents to all intents and purposes appear normal, even with respect to staining properties. Such tissue may then be used to form normal tissue cultures. It will be of fundamental interest to ascertain whether the biochemical properties of the adapted cells are the same as those of normal cells, particularly with respect to the enzymatic properties of the mitochondria.

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[1] Cytological examination was kindly performed by Professor T. E. Weier of the University of California.

The entire question of the effect of the environment on the structure and biochemical characteristics of mitochondria *in situ* assumes particular importance with respect to physiological experiments in which the experimental material is washed in water for hours or days prior to experimental treatment. The washing of disks from tuberous roots or stems is normally carried out for prolonged periods as a preliminary to studies of salt accumulation by these disks (Robertson, 1951; Robertson *et al.*, 1951). That such washing effects both the respiration and the salt-accumulating characteristics of the tissue has been recognized (Stiles and Dent, 1946). The question raised need not be confined to cases of experimental washing, since in nature both fleshy and fibrous roots are normally in hypotonic surroundings. Salt respiration has been linked to the activity of the cytochrome-cytochrome oxidase system (see Robertson, 1951). The latter system has in turn been shown to be exclusively associated with the mitochondria (Hogeboom, 1951). It therefore seems reasonable that the effect of the environment upon the mitochondria may have profound consequences upon certain physiological activities. If the phosphorylative reactions accompanying the respiratory oxidations should prove more directly related to salt uptake than the functioning of the cytochrome system *per se* (Robertson *et al.*, 1951), the effect of low ambient tonicity upon the mitochondria will be of still greater consequence, since the phosphorylative systems have been shown to be the first to be destroyed with any change in mitochondrial structure (Green, 1951). Growth studies must also be concerned with changes in mitochondrial characteristics since growth is almost certainly dependent upon oxidative phosphorylation (Bonner, 1949). Since in nature many tissues are normally in a hypotonic environment, the findings of Buvat (1953) are of particular interest as a possible explanation of the manner in which tissues survive such exposure.

In experiments described elsewhere (Laties, 1953*b*) it has been demonstrated that in cauliflower buds some of the injury to the mitochondrial respiratory systems observed in hypotonic homogenates must be attributed to the action of certain soluble protoplast components. With this in mind it appears possible that the consequence of hypotonic washing of whole tissues may be more drastic with cauliflower tissue than with other tissues. The potato succinoxidase system was found not to be affected by suspension of potato disks in distilled water. However, the succinoxidase system is the least sensitive of the mitochondrial oxidases and the possibility remains of the ubiquitous occurrence of injury to mitochondria upon exposure to water. An effect of washing on potato disks is indicated in experiments carried out by Dr. Helen Sharpensteen which indicate that when disks are cut from potato tubers and aerated in distilled water at room temperature, the respiration increases with time, whereas when aeration is carried out in ice-water, no respiratory rise is noted. The respiratory rise following incubation at room temperature may be achieved at once with freshly cut disks by the use of 2:4-dinitrophenol (DNP). Since, when the respiratory rise due to incubation in water has once taken place, DNP is without further effect, there is some indication that the washing procedure effects an uncoupling similar to that brought about by DNP (Teply, 1949).

The nature of the endogenous competitive inhibitor of succinic dehydrogenase

The irreversible injury which may be suffered by the mitochondria, either as a consequence of hypotonicity *per se* or by action of soluble protoplast components in hypotonic solution, is distinct from the specific inhibition of succinic dehydrogenase brought about by the competitive inhibitor in cauliflower juice. Although this inhibitor has not been identified, the likeliest possibility appears to be that it is a dithio compound of some kind. Dithio compounds may be split by the addition of H_2O, bisulphite, or HCN, in the latter instance with the production of one mole of sulphydryl compound and one mole of a thiocyanate. An inhibition due to a dithio compound may thus be relieved by the addition of HCN. Oxalacetate, which may also be removed by cyanide addition, would not be expected to survive boiling.

Ames and Elvehjem (1944) have reported the inhibition of succinoxidase by cystine, the latter bringing about the oxidation of sulphydryl groups essential to the activity of the enzyme. Such inhibition is lessened when the addition of succinate precedes that of cystine. Furthermore, the presence of either fumarate or malate lessens the inactivation effected by cystine, ostensibly by protecting the vulnerable sulphydryl group(s) of succinic dehydrogenase. Thus when a dithio inhibitor is added either together with or following succinate, the extent of inhibition observed will depend on the ratio of succinate to inhibitor, much as in the case of a true competitive inhibitor. Although protection of this kind by substrate would be expected to diminish with time, the time in which such diminution is observed would be considerably extended if endogenous systems exist which can regenerate oxidized sulphydryl groups, albeit not as quickly as they would be oxidized by cystine in the complete absence of succinate. In the relatively short-time experiments described above the competitive aspects of a dithio inhibitor could be explained in this manner.

The removal of cellular materials by tissue washing

Yet another consideration which must be recognized in experiments involving the washing of whole tissue pertains to the removal of materials from the cells. It is not at all clear to what extent substances may be washed from whole cells. Although glucose has been reported to escape readily from brain slices in the absence of all salt (Elliott, 1946), neither plant, microbial, nor animal cells are normally rapidly depleted of endogenous substrates by washing of short duration. However, there are many indications that a variety of compounds may escape from intact cells into the surrounding medium. Elliott (1940) has described an inhibitor of succinoxidase found to a great extent in certain tumour tissues, which inhibitor diffuses out from tissue slices into the surrounding medium. Such an observation supports the possibility that the washing of cauliflower buds in fact may remove the competitive inhibitor described herein.

Lundegårdh and Stenlid (1944) have reported the loss of nucleotides and flavanone from living roots; Virtanen and von Hausen (1951) have described the appearance in soil solution of dicarboxylic amino-acids from the roots of legumes; and Mirsky and Pollister (1942) have indicated that large quantities of both protein and nucleic acid may be washed from tissue slices without destroying the apparent outlines of the cells. Although the leakage of salts from carrot root tissue into distilled water has been shown to be trivial even when the accumulating mechanism has been made inoperative (Robertson *et al.*, 1951), Humphries (1951) has demonstrated the ready loss of both inorganic salts and organic nitrogen from excised barley roots aerated in nutrient solution. Furthermore, Steward *et al.* (1943) described the loss of potassium from potato disks as a consequence of washing. The possibility of leaching must therefore be considered in experiments involving prolonged tissue washing. With respect to the loss of any given substance, such loss may be exaggerated in salt solution if the substance under consideration is ionic in nature, and exchange is possible (Davson and Danielli, 1952).

Many respiratory measurements tacitly assume bidirectional permeability at least to certain artificial respiratory electron carriers. For example, the manner in which methylene blue is frequently utilized must be taken to indicate that reduction of the dye is accomplished intracellularly followed by the diffusion of the leuco form into the surrounding solution, since the reintroduction of air to the system results in the immediate return of colour to the solution. By the same token it would appear that ferrocyanide at least may freely leave the cell. Although the manometric manifestation of ferricyanide reduction may be explained by the reduction of ferricyanide within the cell, with the subsequent transfer of H^+ to the solution, the potentiometric measurements of ferricyanide reduction depend upon the presence of ferrocyanide in the outer solution. Hochster and Quastel (1952) have made use of this ability of ferri- and ferrocyanide to enter and leave the cell respectively, in experiments in which the ferri- ferrocyanide system was used as a shuttle to carry electrons from within the cell to the insoluble MnO_2 outside the cell. This system, which may be used anaerobically without completely by-passing the phosphorylative mechanism (Cross *et al.*, 1949), may prove useful in further elucidating the relationship between respiration and salt accumulation.

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Enzyme Changes in Relation to Cell Growth in Excised Root Tissues

E. ROBINSON AND R. BROWN[1]

Botany Department, Leeds University

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SUMMARY

Serial sections 1·0 mm. in length taken from the tip towards the base of the bean root have been cultured on 2 per cent. sucrose. At various time-intervals, length, invertase, phosphatase, and protein content of the sections have been determined. Alterations in the enzyme complement of the sections have been related to growth and protein content. The relation of changes occurring in excised fragments to those in the intact root have been discussed.

INTRODUCTION

In an earlier paper it was shown (Robinson and Brown, 1952) that expansion of the cell in the intact root is accompanied by extensive changes in the enzyme complement and it was suggested that growth is controlled by these. If such is the case, however, changes should also occur during the culture of tissue excised from the growing zone of the root and their characters should vary with the extent to which growth occurs. Sections excised from certain regions of the growing zone expand in culture as a result of cell growth while those from other regions do not. Clearly if growth is controlled by changes in the enzyme complex, then the nature and extent of such changes might be expected to vary with the capacity for growth. The present investigation has been undertaken to examine this possibility.

The experimental material consists of 1·0 mm. sections cut successively from the apex of the root to a distance of 10·0 mm. Length, phosphatase, and invertase activities are measured on corresponding groups of sections after various periods of culture. In certain instances the protein content has also been determined. Brown, Reith, and Robinson (1952) showed that growth in the first two sections is limited, that it is vigorous in the third and fourth, and again limited in the fifth and subsequent sections of the series. It may be emphasized that when increase in length of the sections occurs it is entirely due to the growth of the component cells. Thus length and enzyme activity measurements for the different sections provide the basis for a comparison between activity changes and cell growth in culture. The protein determinations have been incorporated in the experimental design since it was shown in the earlier investigation that in the intact root changes in enzyme activity may be related partly to the change in total protein content.

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[1] Present address: Unit of Plant Cell Physiology, Department of Agriculture, Oxford.

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Observations on enzyme activity have been restricted to the invertase and phosphatase systems since these are readily estimated and also in preliminary experiments it was found that each changes differently, in that one tends to increase and the other to decrease in culture. It is recognized that the two systems which have been examined may not immediately control the growth process. The primary purpose of the investigation, however, is the examination of the possibility that changes in the enzyme complement accompany growth in culture. Such changes, if they occur, are probably due to modifications in the structure of the protein which would affect the two systems studied together with all other components.

MATERIAL AND METHODS

Beans (var. *Aquadulce*) are grown under the controlled conditions, and sections cut from roots of standard length, by means of the technique described previously (Robinson and Brown, 1952). The experimental material consists of a series of ten 1·0 mm. sections cut from the tip towards the base of the root. The sections are cultured on coarse sintered glass disks placed in Petri dishes containing 2 per cent. sucrose in groups of 10 for invertase estimations or 20 for phosphatase estimations. The diameter of the dishes is 3·0 cm. and of the disks 2·0 cm. Two millilitres of sucrose is added to each dish. This volume of fluid ensures that the upper surface of the disk is moist, but not covered with free liquid. The cultures are incubated for various periods of time at 25° C. in the dark. The culture technique is based on that described by Brown, Robinson, and Johnson (1950).

At the end of the culture period the dishes are withdrawn from the incubator and the length of each section measured using a calibrated micrometer eyepiece on a microscope. From each set of measurements a mean volume is calculated and these values are given in the next section. After the length measurements have been made the fragments are used immediately for the estimation of either phosphatase or invertase. The techniques used for estimation of enzyme activity are those described by Robinson and Brown (1952). Phosphatase activity is measured as the rate at which phosphate ions are released from a buffered solution of sodium β-glycerophosphate and invertase activity as the rate of hydrolysis of sucrose. The substrate for the invertase estimations is unbuffered. In an earlier investigation a comparison was made of the invertase activity of sections placed in sucrose buffered at various pH values. It was found that optimum values were obtained at pH 6·0 and that these were approximately the same as those obtained with a sucrose solution in distilled water (pH 6·0 approx.) (Robinson and Brown, 1952).

The various estimations have been made after 6, 12, 24, 36, and 48 hours of culture. It has been found convenient to obtain one set of results for one cultural period for the whole series of sections. Thus the results for different cultural periods have been obtained with different batches of roots handled on different occasions. The variation in enzyme activity given by different batches of roots is high, and accordingly each experiment has been repeated on three different occasions. The enzyme values quoted in the next section are each the mean of three determinations.

In addition to length and enzyme activity measurements, protein estimations have also been made. A separate set of 100 sections has been used for each of the protein estimations. Each set has been cultured on a coarse sintered glass disk 7 cm. in diameter placed in a 9·0 cm. Petri dish containing 10 ml. of 2 per cent. sucrose. After different cultural periods the sections have been removed, ground to a paste, and the protein content determined by the technique described in the earlier investigation (Robinson and Brown, 1952).

The different sections of the experimental series are referred to below as $S_1, S_2, \ldots, S_{10}$. The subscript shows the number of the section in the series and also indicates the distance in mm. of the basal edge from the tip of the root at the time of excision.

EXPERIMENTAL RESULTS

1. *Length measurements.* The average lengths of the fragments after different cultural periods are given in Table I.

TABLE I

Average lengths (mm.) of sections after various periods of culture on 2 per cent. sucrose. Figures at heads of vertical columns represent time in hours

	0	6	12	24	36	48
S_1 . .	1·0	1·09	1·12	1·07	1·12	1·36
S_2 . .	1·0	1·12	1·19	1·48	1·30	2·49
S_3 . .	1·0	1·22	1·38	1·74	3·35	3·39
S_4 . .	1·0	1·29	1·78	2·98	3·38	3·33
S_5 . .	1·0	1·26	1·30	1·87	2·09	2·39
S_6 . .	1·0	1·16	1·11	1·44	1·66	1·79
S_7 . .	1·0	1·15	1·08	1·07	1·17	1·61
S_8 . .	1·0	1·06	1·06	1·07	1·09	1·37
S_9 . .	1·0	1·05	1·04	0·99	1·03	1·24
S_{10} . .	1·0	1·05	1·02	1·00	1·04	1·24

In an earlier paper (Robinson and Brown, 1952) it was shown that the individual cells of intact roots grown under conditions identical with those used for this investigation increased in length over the first 8·0 mm. from the tip of the root. The changes in length observed in excised sections in culture correspond to this observation. All sections up to S_8 show some increase in length. Clearly the increase in length of the 9th and 10th fragments is negligible. Thus the results indicate that the increase in length after excision is related to the potential increase that may still occur in the parent root.

The greatest extension occurs in sections 3 and 4, where the increase in length is greater than threefold. Further it may be noted that maximum length is reached in S_4 in 24 hours, in S_3 in 30 hours, and in S_2 not until 48 hours have elapsed. In S_2 there is an abrupt acceleration of growth between 36 and 48 hours.

2. *Change in invertase activity.* The average invertase activity of the sections after various cultural periods are shown in Table II.

TABLE II

Invertase activity (g. × 10⁻⁵ reducing sugar produced per section per hour) of sections after various periods of culture on 2 per cent. sucrose. Figures at heads of vertical columns represent period of culture in hours. Figures in parentheses indicate relative enzyme activity per cell

	0		6	12	24	36	48
S_1	3·00	(1·19)	3·6	3·1	2·4	3·1	2·6
S_2	4·90	(1·0)	5·4	4·4	5·0	6·0	6·0
S_3	12·9	(2·69)	14·5	8·3	10·0	11·1	5·8
S_4	16·1	(5·29)	25·2	6·1	11·7	3·5	5·5
S_5	22·1	(10·54)	12·5	5·5	5·7	4·1	3·9
S_6	22·3	(12·93)	9·5	7·3	5·7	3·7	3·6
S_7	16·6	(13·12)	15·1	4·2	4·1	3·2	4·2
S_8	14·0	(15·29)	14·1	3·3	4·0	2·8	3·7
S_9	11·0	(11·36)	17·7	3·3	3·4	3·7	3·6
S_{10}	13·3	(14·44)	11·9	3·3	3·2	4·4	3·4

The figures in parentheses in Table II show the relative invertase activity of the average cell in each section at the time of excision, and thus they indicate how activity changes with growth of the cell in the intact root. These data are similar to those given in a previous paper (Robinson and Brown, 1952). Both sets of data show that the activity remains more or less constant in S_1 and S_2 and then increases as the cells expand up to S_8: the activity then decreases.

The present series of results show that in the first two sections there is little change in invertase activity during the culture period. The final value (48 hours) for S_3 and S_4 is lower than the initial value. In all subsequent sections invertase activity decreases sharply during the first 12 hours and then remains more or less constant. In these sections the final value is much lower than the initial. In S_1–S_4 there is some indication that activity increases during the first 6 hours.

3. *Change in phosphatase activity.* The average phosphatase activity of sections after various periods of culture on 2 per cent. sucrose are shown in Table III.

The figures in parentheses (Table III) show the relative phosphatase activity per cell in the successive sections immediately after excision. They demonstrate the same changes as recorded in an earlier report (Robinson and Brown, 1952). The relative activity decreases from the 1st to the 2nd section, increases from the 2nd to 8th, and then decreases. In culture, the phosphatase activity of the 1st and 2nd sections remains more or less constant, that of the 3rd and 4th probably increases slightly, and of the 5th and subsequent sections increases markedly, particularly within the first 12 hours. It may be noted that in the 1st and 2nd sections in which growth is limited, there is little change with time in either phosphatase or invertase activity. In

TABLE III

Phosphatase activity (g.$\times 10^{-4}$ P released per section per 4 hours). Figures at heads of vertical columns represent time in hours. Figures in parentheses show relative activity per cell

	0	6	12	24	36	48
S_1	3·6 (1·63)	3·5	3·1	2·5	2·9	3·0
S_2	4·2 (1·00)	4·0	4·2	3·5	3·5	3·9
S_3	4·5 (1·08)	4·1	4·4	4·4	4·4	5·5
S_4	3·7 (1·40)	3·8	4·2	3·4	4·5	3·3
S_5	3·5 (1·92)	3·5	3·8	3·6	4·1	3·3
S_6	2·7 (1·88)	3·8	3·5	3·1	3·6	3·4
S_7	2·3 (2·08)	2·9	2·9	2·3	2·9	3·1
S_8	2·1 (2·63)	2·6	2·6	2·1	2·8	2·6
S_9	2·0 (2·38)	2·5	2·6	2·4	2·8	2·6
S_{10}	1·9 (2·35)	2·6	2·5	2·2	2·6	2·6

the 3rd and 4th sections where growth is vigorous there is a slight decrease in invertase and a slight increase in phosphatase. In the 5th and subsequent sections in which growth is again limited invertase activity decreases sharply and phosphatase activity shows a corresponding increase.

4. *Change in protein content.* The protein contents in two representative sections cultured for various periods of time on 2 per cent. sucrose and on water are shown in Table IV.

TABLE IV

Protein-N contents (g. 10^{-5}) per section after different cultural periods on 2 per cent. sucrose and on water. Figures at heads of vertical columns represent time of culture in hours

	0	6	12	24	36	48
	2 per cent. sucrose					
S_3	1·58	1·58	1·42	1·40	1·41	1·23
S_{10}	0·57	0·56	0·59	0·53	0·52	0·48
	Water					
S_3	1·69	—	—	1·49	—	0·98
S_{10}	0·57	—	—	0·48	—	0·44

It is evident that on 2 per cent. sucrose there is little change in protein content during the first 36 hours of culture. There may be a slight decrease between 36 and 48 hours. In sections cultured on water, protein content decreases progressively with time.

DISCUSSION

It is evident from the data presented above that extensive changes may occur in the enzyme complement of excised tissues cultured on 2 per cent. sucrose. Further, these changes cannot be referred to corresponding changes in the protein content. Thus while protein content remains constant or decreases slightly phosphatase activity increases, and while a slight decrease in protein content occurs between 36 and 48 hours, the most striking change in invertase activity occurs within the first 12 hours of the cultural period. It may be emphasized that the changes in the enzyme activity are certainly not the effects of moribund conditions in the tissues. The greatest changes in the activities of the two enzymes occur in the first 12 hours of culture when growth is still proceeding and respiration is vigorous.

The observed changes in enzyme activity may be due to the development of an inhibitor for invertase and an activator for phosphatase. It is significant, however, that in all sections in which changes occur the increases and decreases are more or less simultaneous. This suggests that they are the expression of fundamental developments in the composition of the cytoplasm, and that they probably represent changes in the structure of the protein complex of the cell. It is possible that during culture the qualitative composition of the protein changes in such a way that a component which represents invertase decreases and that another which represents phosphatase increases.

Such generalized changes are clearly not simply effects of excision. Similar developments occur in intact organs. It has been shown (Robinson and Brown, 1952) that when cell growth occurs in the intact root not only do enzyme activities alter with variations in the absolute content of protein, but they also change relative to each other and to the protein level. Relative changes become absolute changes when there is little variation in the protein. It is therefore probable that the absolute changes observed with the cultured fragments are of the same general character as the relative changes that have been described with the intact root. It is posssible that while protein increases as the cell expands the composition of the whole complex changes in such a way that the proportion of an invertase component increases while that of a phosphatase component remains constant.

At the same time it may be noted that if it is indeed changes in the nature of the protein that determines absolute changes in enzyme activity in the excised frequent and relative changes in the intact root, then the qualitative changes are not the same in all components of the protein complex. In certain components similar changes probably occur. With invertase activity there is a tendency for the same changes to occur after excision that continue when the cells are in the root. With the first fragments there is an increase in activity during the first 6 hours which no doubt corresponds to the relative increase that has been observed in the intact root during the development that proceeds over the first 6 mm.

It is significant that enzyme activity (at least with respect to invertase and phosphatase) does not change to the same extent in all fragments during culture. With S_1 and S_2 there is little or no change and a relatively small change occurs in S_3 and S_4. The greatest changes are observed in the fifth and subsequent fragments of the series. Clearly the data suggest that the whole system in the successive fragments becomes progressively more labile. They suggest that it changes in such a way that the protein becomes progressively more readily transformed as the succession from the first to at least the eighth section is traversed. At the same time since enzyme activity does not change in some sections of the series it is clear that the transition that occurs in the intact root from the state of one fragment to that of the next does not proceed when the tissue is excised. It is probable that the transition requires the provision of particular metabolites which are supplied from mature tissue. These metabolites are not available in the tissue after excision and the state of relative stability may therefore be retained throughout the cultural period. This interpretation is consistent with that proposed by Brown and Wightman (1952) and by Robinson and Brown (1952), who have suggested that many of the metabolites required in the meristem cannot be synthesized in the apex and are translocated from mature regions of the root. It may be noted that the first two fragments in which the system is apparently relatively stable are those in which cell division occurs and they are also those in which the amino-acid composition of the protein differs from that in other parts of the root (Brown, Reith, and Robinson, 1952).

Brown, Reith, and Robinson have suggested that growth in an excised fragment is determined by the activity of a particular enzyme complex, that this complex decreases in quantity with time, and that the decrease in the rate of growth with time is an expression of this change. The present series of data show that decreases in the levels of particular enzymes may indeed occur in isolated fragments in which growth proceeds. At the same time two important features may be noted in this connexion: growth is limited in the first two fragments in which there is little or no change in activity with time; although growth only occurs in fragments in which some change in activity is observed, nevertheless vigorous growth only occurs in the third and fourth fragments in which activity changes are less pronounced than they are in more mature tissues in which change in length in culture is restricted.

It has been suggested above that the transition from the second to the third section involves a development to a more labile state. As the system becomes more unstable the possibility for the synthesis of new enzymes increases, and it is probable that in the transition from the second to the third fragment enzyme systems are developed which promote growth. Growth, however, is likely to be most extensive when the rate of protein transformation is lowest. With each successive fragment the system becomes more labile, and the specific complex involved in growth becomes progressively more unstable, with the result that growth becomes progressively more restricted. With the third and fourth fragments the system is relatively stable, the rate of transformation is low, and growth is consequently more vigorous.

The interpretation developed above is based on the final values for lengths and activities after 48 hours. It is clear, however, that if growth depends on a particular enzyme complex, then the time during which growth proceeds should vary with the rate of enzyme transformation. This aspect of the relation is not displayed by the data provided above, probably because the systems studied may not be immediately involved in growth, and the measurements of activity are necessarily crude. Some indication, however, of a connexion between rate of transformation and time of growth has been obtained in another series of experiments.

Brown and Sutcliffe (1950) showed with maize root segments that the time during which growth proceeds increases with increase in concentration of sugar in the culture medium. When sugar is not supplied growth ceases within 12 hours, with 0·25 per cent. sucrose it continues for 24 hours, with 2 per cent. sucrose for 36 hours, and with 5 per cent. for at least 48 hours. A similar effect has been demonstrated with bean roots. In this connexion it is of some significance that the rate at which invertase activity decreases varies with the concentration of sucrose in the culture medium. Invertase activity has been determined after 24 and 48 hours on different concentrations of sucrose with two fragments. The results are shown in Table V, the values of which are each the mean of 4 from separate determinations.

TABLE V

Invertase activity after culture for 24 and 48 hours on different concentrations of sucrose. Figures at heads of vertical columns are percentage concentrations of sucrose

	Time (hours)	0	2	4	6	8
S_4 . .	24	2·15	6·97	9·03	9·75	9·96
	48	2·76	5·07	6·73	8·65	8·83
S_8 . .	24	2·15	3·93	4·41	5·23	6·45
	48	2·15	3·93	4·04	4·74	5·16

Invertase activities tend to increase as the concentration of sugar in the external medium increases. Clearly with increasing concentration the rate at which invertase is transformed into some other protein decreases. This result does not of course show that the increase in the time of growth depends on a decrease in the rate of invertase transformation. It does suggest, however, that it may depend on the rate of decrease of some other enzyme which catalyses a reaction in which sugar is involved. The results suggest that sugar may exert a protective action not only on invertase but also on an enzyme immediately involved in growth, and that the increase in the time of growth is due to a depression in the rate at which this enzyme is transformed into another protein.

It may be noted that the effect of external sugar concentration on the level of invertase tends to support the interpretation that the change in activity represents a change in the composition of the cell proteins.

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Polyphenoloxidase and the Respiration of Ivy Leaves

NATALIE H. LERNER

Botany School, Cambridge[1]

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SUMMARY

1. Polyphenoloxidase is present in ivy leaves but not in those of *Aucuba* or *Euonymus*.

2. Respiration of intact ivy leaves was markedly stimulated by catechol (R.Q. approximately $= 1$), gallic acid, caffeic acid, and dihydroxyphenylalanine. The stimulation was not related to injury as far as could be detected and was not followed by inhibition. The extra oxygen consumption represents a many times repeated oxidation of the amount of catechol supplied.

3. The effect of catechol on the respiration of *Aucuba* and *Euonymus* leaves was very small.

4. Cupferron and phenylthiourea, which inhibit polyphenoloxidase *in vitro*, nevertheless increased respiration when administered to leaves through the petiole. On the other hand, when applied by infiltration, cupferron did cause inhibition, but in *Aucuba* and *Euonymus* as much as in ivy.

INTRODUCTION

THE function of the enzyme polyphenoloxidase in plant cells has been discussed by many authors. A theory that has found some favour is that polyphenoloxidase is a terminal oxidase of respiration. Boswell and Whiting (1938), working with washed potato slices, were the first to bring any experimental evidence in support of this theory, and later Baker and Nelson (1942), while disagreeing in part, supported their conclusion. The enzyme has been claimed as the terminal oxidase of sweet potato roots (Walter and Nelson, 1945) and of spinach leaves (Bonner and Wildman, 1946). Schrade, Bergman, and Byer (1948), however, after a careful study, have concluded that polyphenoloxidase is not part of the normal respiratory mechanism of the potato.

An alternative suggestion that the enzyme plays a part in photosynthesis has been made by Arnon (1949), following the discovery by Warburg and Lüttgens (1946) that quinones will stimulate the production of oxygen by isolated chloroplasts, and that the polyphenoloxidase in spinach leaves is found only in the chloroplasts.

The present investigation examined the possibility that polyphenoloxidase might be the terminal oxidase of respiration in the ivy, *Hedera helix* Linn.

From the extensive *in vitro* studies that have been made, which are reviewed by Nelson and Dawson (1944), it is known that polyphenoloxidase is

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[1] Now at the University College of the Gold Coast.

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a copper-containing protein having a variety of substrates. The enzyme oxidizes dihydroxyphenols *in vitro* in two stages with the uptake of two atoms of oxygen per molecule of phenol. The first stage, involving the uptake of one atom of oxygen, results in the formation of a quinone. It is thought that in plant cells the quinone formed is then reduced by the cell dehydrogenases the dihydroxyphenol thus acting as an electron carrier. The fate of the second atom of oxygen taken up *in vitro* has been discussed by Mason, Schwartz and Peterson (1945). Robinson and Nelson (1944) have suggested that in potato tubers dihydroxyphenylalanine acts as the natural carrier for the polyphenoloxidase present in that tissue.

If polyphenoloxidase is functioning as a terminal oxidase of respiration then addition of a suitable carrier would be expected to increase the rate of oxygen uptake. The percentage increase might only be a small one if either the combination of the oxidase with oxygen, or the combination of the dehydrogenases with the hydrogen donator reducing the carrier, were the main rate-determining reactions.

In the present work observations have been made on the effects of adding a number of dihydroxyphenols to ivy leaves, and in addition attempts have been made to obtain inhibition of the polyphenoloxidase *in vivo*. The investigation has been confined to physiological observations on the living tissues

EXPERIMENTAL METHODS

In most experiments whole leaves were used. Continuous records of carbon dioxide production were obtained by the Pettenkofer technique using a Blackman commutator and two- or four-hourly periods. At selected points in the course of these records of the drift of the carbon dioxide output determinations were made of the respiratory quotient on the same material using a method and apparatus developed by Contreiras (1949). Respiratory chambers used were wide-mouthed glass jars having ground-glass stoppers fitted with inlet and outlet tubes and a capillary manometer. The internal volume of the chambers was 350 ml. and they were covered with bags of black cloth and immersed in a water-bath at 23° C. Before passing into the experimental chambers the air-stream was passed through a water-vapour saturator immersed in the bath.

When working with whole leaves dihydroxyphenols or inhibitors were introduced through the petioles. A separate glass vial was used for each leaf, the petiole being passed through the narrow opening and dipped into the solution in the vial. To obtain rapid absorption the leaves were then exposed to the light in flat glass-sided chambers through which a stream of carbon dioxide-free air was passed; controls, supplied with water only, were similarly treated. The leaf samples of about 10 leaves weighing 10–15 g. were weighed before and after this procedure. Only experiments where the weight remained constant or nearly so were carried farther. The amount absorbed was estimated for each leaf by weighing leaf plus vial before and after exposure so that for experiments leaves could be chosen that had absorbed approximately equal amounts. Mean concentrations in the leaf were estimated from the amount of solution taken up and the mean moisture content of the leaves (usually about 65 per cent. of the fresh weight).

Carbon dioxide production by all the leaf samples was usually recorded for 20–40 hours before dosing (pre-dosage period). After dosing, samples were returned to the bath and a 2-hour period was allowed for equilibration before resuming records of carbon dioxide production. Some experiments were also carried out on cut pieces of leaf tissue in a Warburg apparatus. In these cases the leaves were immersed in the chosen solutions in the vessels, evacuation followed by release of pressure being repeated until translucence indicated complete injection of the air spaces.

EXPERIMENTAL RESULTS

(*a*) *The effect of catechol in air.* A number of experiments were carried out using a range of catechol concentrations. Typical records of carbon dioxide production in such experiments are shown in Fig. 1. It will be seen that following the dosing period the rate for the water control rises. The extent of this rise varies with the season and the light conditions, but it will be seen that catechol, even in concentrations as low as 0·0033 M., produces an increase in respiration over the water controls. In the case of the higher concentrations used this increase is maintained over many hours, and no instance of inhibition was observed.

TABLE I

The effect of catechol on CO_2 *production*

Expt. No. and date	Catechol molarity		Percentage stimulation		Total extra CO_2 mg./g. F.W.	Duration of expt. hours	No. of times catechol oxidized and reduced	
	Supplied	In leaf	0–24 hrs.	Remainder of expt.			Over total	Per hour
1*	0·004	0·0023	0	—	—	—	—	—
4/12/46	—	0·0021	0	—	—	—	—	—
49	0·0025	0·002	6	2·5	0·308	40	5·9	0·15
27/12/48	0·0033	0·0024	32	15	1·676	—	26·2	0·66
	0·005	0·0037	77·5	66·5	4·652	—	47·7	1·19
2*	0·005	0·0053	0	−7	—	—	—	—
13/12/46	0·0075	0·0071	49	—	2·384	36	12·5	0·35
	0·01	0·0105	48	1	2·340	—	8·1	0·225
50	0·005	0·0058	143	—	4·163	—	8·5	0·53
10/12/48	0·01	0·012	274	—	7·951	16	16·5	1·03
	0·02	0·018	232	—	6·79	—	14·1	0·88
3*	0·0075	0·0043	56	56	5·134	—	41·8	0·97
3/2/47	0·01	0·0075	80	125	8·577	43	48·1	1·12
	0·02	0·014	53	67	4·922	—	12·2	0·28

* Denotes R.Q.s measured and found not to differ materially in control and treated.

This marked and maintained stimulation of respiration rate by catechol contrasts with the results of Boswell and Whiting (1938) for potato tissue. They found that when catechol, to a concentration of 0·004 M., was added to washed potato slices in phosphate buffer at pH 4·5, there was an immediate rise in carbon dioxide production and oxygen uptake, but that after 20 minutes the rates had fallen below the control, finally reaching a value about one-third of the control level. Baker and Nelson (1942) found a similar effect, but in their experiments the fall was even greater, the final rate being only 15 per cent. of the control rate.

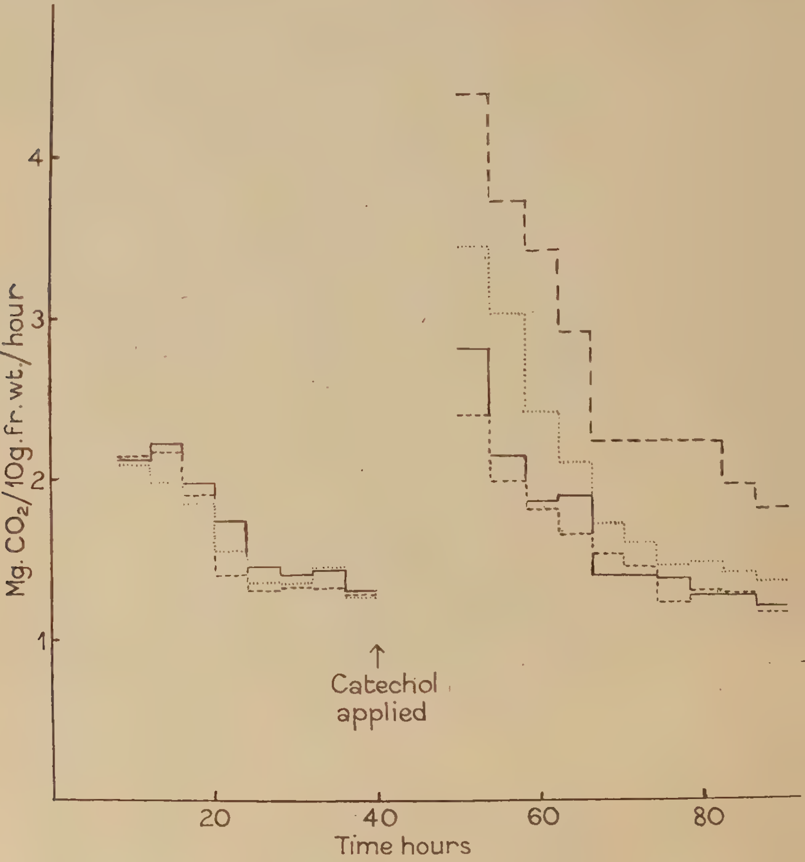

FIG. 1. The effect of catechol on the carbon dioxide production of ivy leaves. Expt. 49. — — — 0·005 M. catechol, ... 0·0033 M. catechol, —— 0·0025 M. catechol, – – – – – – water.

The percentage stimulation found over the first and second 24 hours in a number of the experiments with ivy leaves is given in Table I. Since the amount of solution absorbed was not the same in all experiments, the comparison is best made on the basis of the final estimated concentration in the leaf. Increases in concentration of catechol up to 0·01 M. (except in Expt. 2, Table I) results in an increase in percentage stimulation. Increase in catechol concentration beyond 0·01 M. results in a percentage stimulation only equal or less than that found for 0·01 M. catechol. There appears to be a threshold below which there is little stimulation. Usually this is about 0·002 M., but in an early experiment it was as high as 0·0033 M.

It should be noted that response to catechol is not dependent on observable damage to the leaf tissue. When high concentrations (0·01 M.) are used the veins may become blackened and this may spread to the adjacent mesophyll tissue, but with solutions of 0·005 M., damage rarely occurred, while the stimulation obtained using this concentration was considerable.

The data given so far are for carbon dioxide output only. It is, however, clear that there is an equivalent stimulation of the oxygen uptake since, in all experiments in which the respiratory quotient was measured, no consistent difference was found between control and treated samples.

In Table I experiments in which the R.Q. was measured are marked with an asterisk, and actual figures from one experiment are given in Table II, the times given being mean times after first dosing.

TABLE II

Effect of catechol on R.Q.

	Catechol concentration supplied			
Time (hours)	H_2O	0·0075 M.	0·01 M.	0·02 M.
2	0·98	0·99	1·0	0·99
26	0·95	0·89	0·91	0·90
28	0·93	0·89	0·91	0·91
30	0·89	0·82	0·92	0·89
After a second dosing				
51	1·0	0·91	0·98	0·98
66	0·97	0·94	0·93	0·89

In view of a possible effect of catechol on photosynthesis, it was thought desirable to dose the leaves in the absence of light in case the effect on respiration was merely a secondary one. It proved extremely difficult to make the leaves absorb the solution, but in the one experiment that was successful a stimulation was found. The amount absorbed was small, the concentration supplied was 0·01 M., the final leaf concentration was 0·006 M., and the stimulation 25 per cent. As can be seen from Table I, this is lower than that found for leaves dosed to this concentration in the light. In the light the dosing took 5 hours and in the dark 20 hours, so the experiments are not comparable.

If in these experiments an average R.Q. of 0·94 over the whole period is assumed, we can estimate the total oxygen uptake and carbon dioxide output. In all cases where stimulation was shown the actual amount of extra respiration was in excess of one mole of gas per mole catechol absorbed, thus showing that catechol was oxidized and reduced and acted as an electron carrier.

In Expt. 49, Fig. 1, leaves dosed with 0·005 M. catechol reached a final concentration of 0·263 mg. per g. F.W. leaf tissue. The oxidation of this amount of catechol would require 0·0762 mg. oxygen. The actual total extra respiration was 4·652 mg. CO_2 per g. leaf tissue. Over the whole 40-hour period therefore the catechol would have to be oxidized and reduced 47·7 times, or 1·19 times per hour. These rates would be doubled if only one atom of oxygen were taken up on each oxidation. Results for a number of other experiments are given in Table I.

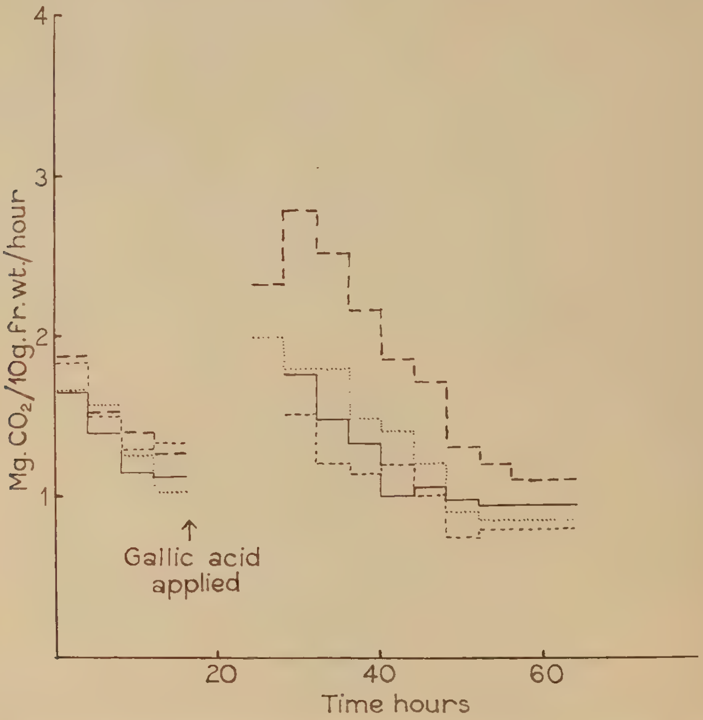

FIG. 2. The effect of gallic acid on the carbon dioxide production of ivy leaves. Expt. 25. ——— 0·01 M. gallic acid, ······· 0·005 M. gallic acid, —— 0·0025 M. gallic acid, - - - - - - water.

(*b*) *Other dihydroxyphenols.* The effects of gallic acid, caffeic acid, and dihydroxyphenylalanine have been investigated. These solutions caused no visible damage to the leaf tissue. The effects of gallic acid and dihydroxyphenylalanine are shown in Figs. 2 and 3 and similar stimulatory effects were found with caffeic acid. All concentrations used increased respiration.

Having established that catechol and other diphenols increase the respiration rate of ivy leaves, the mechanism whereby such an effect is produced has to be explained. It is often assumed that this effect strongly suggests that polyphenoloxidase is a terminal oxidase of respiration. Other explanations are possible. The increase of respiration following dosing with catechol

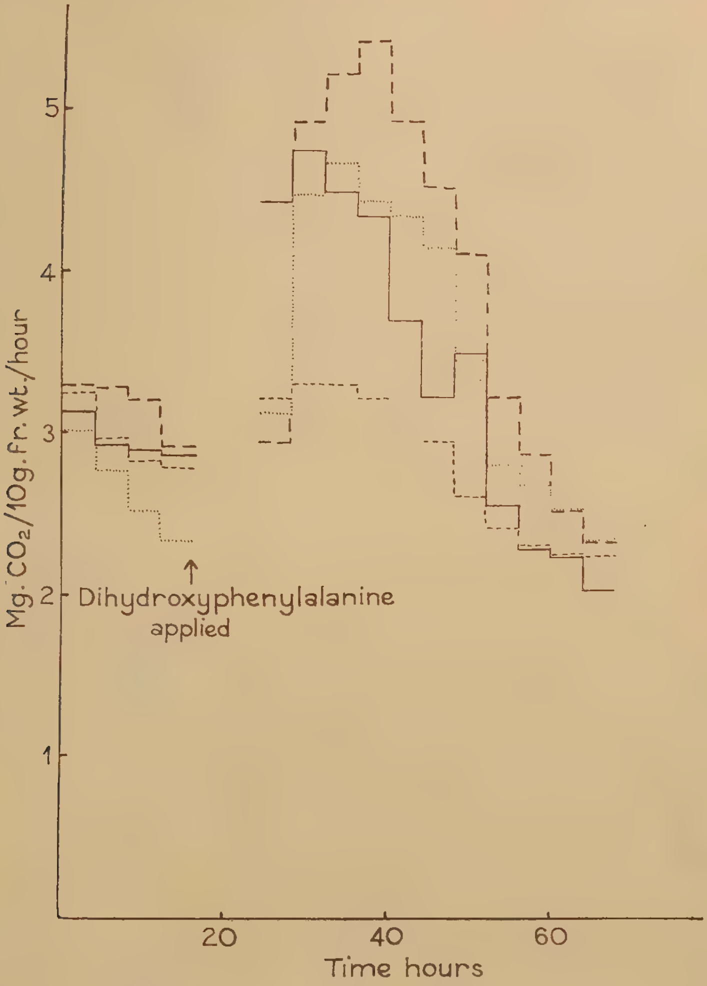

Fig. 3. The effect of dihydroxyphenylalanine on the carbon dioxide production of ivy leaves. Expt. 39. ——— 0·005 M. DOPA, ······· 0·0025 M. DOPA, —— 0·0025 M. DOPA, ------ water.

could conceivably also be due to: catechol causing increased permeability of the cell which could result in increased respiration; catechol stimulating an enzyme in the respiratory cycle other than an oxidase; or to catechol inducing the functioning of polyphenoloxidase which is not normally active in cell respiration.

The first possibility could be eliminated if it were possible to demonstrate that the action of the catechol were found only in leaves containing polyphenoloxidase. Absence of stimulation by catechol under anaerobic conditions would also suggest that stimulation is due to participation of the oxidase system directly. Some information regarding the normal participation of the enzyme system might be obtained by the use of inhibitors.

(*c*) *The effect of catechol on non-polyphenoloxidase leaves.* The search for a broad-leaved evergreen shrub whose leaves did not contain polyphenoloxidase (tested by the guaiacum reaction) was not easy, but eventually two were found, *Aucuba japonica* Thunb. and a *Euonymus* species not yet determined. Neither of these was very satisfactory; both were difficult to dose, as *Aucuba* absorbed very little and wilted easily, while *Euonymus* leaves were small and the petioles short and thin. The respiration rates during the pre-dosage period were often irregular, and the differences between samples greater than in the case of ivy.

In experiments with catechol and caffeic acid at various pH it was found that, while in some cases there appears to be a small stimulation of carbon dioxide production at high (0·02 M.) concentrations, the effect is not of the same order as that found for ivy. A selection of these results are shown in Table III. The percentage stimulation was measured over the first 24 hours.

TABLE III

The effect of dihydroxyphenols on CO_2 *production of* Aucuba

Expt. No. and date	Diphenol	Molarity supplied	Molarity in leaf	CO_2 production in pre-dosage period as % control	CO_2 production after dosing as % control
37 5/7/48	Catechol	0·005	0·0025	116	132
		0·01	0·0055	126	139
		0·02	0·015	102	147
48 10/12/48	Catechol	0·005	0·0036	95	122
		0·01	0·008	102	120
30 22/3/48	Caffeic acid	0·005	0·0033	91	107·5
		0·01	0·0078	98	133
29 17/3/48	Caffeic acid neutralized	0·0025	0·0013	89·5	105
		0·005	0·0025	99	115
		0·01	0·005	98	106·5

Table III, column 5, gives the average respiration rate over the whole of the pre-dosage period for each set expressed as a percentage of the control set. This is a measure of the variability of the untreated leaves. The rates after dosing as percentage of the control rate are all higher than before but the difference is small.

It would appear from these experiments that catechol and caffeic acid have at least two effects on carbon dioxide production. One of these, small in comparison with the total effect of these phenols on ivy leaves, is not due to an effect on the polyphenoloxidase.

(*d*) *The effect on respiration of inhibitors of polyphenoloxidase.* Inhibitors considered to be specific for polyphenoloxidase are phenylthiourea (Dubois and Erway, 1946) and BAL (Webb and van Heyningen, 1947); cupferron and dithiodiethylcarbamate (by combining with the copper atom); *p*-nitrophenol (Bonner and Wildman, 1946) and resorcinol (Richter, 1934) are competitive; carbon monoxide is also specific if administered in the light but therefore cannot be used with photosynthetic tissue. Only cupferron and phenylthiourea were used in Pettenkofer experiments reported here. Both were found to inhibit the guaiacum reaction in crushed ivy leaf tissue at a concentration of 0·01 M.

Cupferron, so far from lowering the respiration rate, was actually found to increase carbon dioxide production. This increase was sustained for many hours. In Table IV are given the results for a number of experiments. The R.Q. of control and treated leaves were again both near unity. The R.Q.s in one experiment, both in air and in 2·5 per cent. oxygen, were found to lie between the values 0·92 and 1:02.

TABLE IV

The effect of cupferron on the CO_2 *production of ivy leaves*

Expt. No. and date	Molarity supplied	Molarity in leaf	CO_2 production in pre-dosage period as % control	CO_2 production after dosing as % control
53 10/3/49	0·0025	0·0014	100	126
	0·005	0·0022	92·1	121
	0·01	0·0075	90·6	156
23 18/12/47	0·005	0·003	107	102
33 19/5/48	0·005	0·0057	98·8	118
	0·01	0·0105	89·8	105
	0·02	0·0183	101	147

It was thought that while cupferron might not inhibit normal respiration, it might inhibit that part of respiration stimulated by catechol. Experiments designed to test this showed that previous dosing with cupferron did not prevent stimulation by catechol.

Phenylthiourea was also used on intact leaf tissue. It was also found to increase the respiration rate even more markedly than cupferron and at concentrations as low as 0·001 M.

In the inhibition tests described above, the inhibitors were introduced via the petiole. In further tests the method of injecting the mesophyll air spaces was used, the rates of oxygen uptake and carbon dioxide output being determined manometrically in a Warburg apparatus. Gas exchange rates are given in μl. g. fresh weight hour for successive hour periods. Injection of the leaves with water increases the diffusion path considerably, and it was found that in order to ensure fully aerobic respiration oxygen concentrations greater than 70 per cent. had to be employed. In all experiments quoted 100 per cent. oxygen was used. The percentage stimulation of respiration by catechol was much lower than in intact leaves. This was considered to be a direct result of the low oxygen tension in the leaf limiting the rate of respiration, as the percentage stimulation increased as the oxygen concentration was increased. Intact leaves previously dosed with catechol and later cut up and injected with distilled water similarly showed a much lower stimulation. The rate of respiration in the catechol-injected leaves was as high as or higher than the rate of respiration in dosed intact leaves; that of water-injected leaves was, however, much higher than undosed intact leaves. This was due to the fact that cutting the leaves into small pieces was found to increase the respiration rate, the R.Q. remaining unity. The increase in respiration was roughly proportional to the length of the cut edge.

The results obtained when cupferron is injected in this way are given below:

	0·01 M. cupferron		Water	
Oxygen uptake . . .	220	197	305	288
Carbon dioxide output . .	257	243	283	248

While there is only a small depression of carbon dioxide output, there is a marked depression of oxygen uptake. If cupferron inhibition of polyphenoloxidase were competitive with oxygen, then the existence of an appreciable inhibition when using injected instead of intact leaves might be ascribable to the low internal oxygen concentration produced by the high diffusion resistance of the water in the intercellular spaces. This explanation is rendered unlikely by the fact that no inhibition appeared in leaves dosed through the petiole in external oxygen concentrations as low as 2·5 per cent.

Aucuba and *Euonymus* leaves injected in this way with cupferron show this same inhibition; the rates for *Aucuba* were as follows:

	0·01 M. cupferron				Water			
Oxygen uptake	149	139	145	142	221	220	223	213
Carbon dioxide output . . .	179	177	175	164	196	204	232	243

The absence of any inhibiting effect by cupferron on respiration of intact leaves suggests that polyphenoloxidase does not take part in their respiration. The cupferron inhibition of injected leaves suggests that their respiration differs in its mechanism from that of intact leaves. As cupferron has been shown to be non-specific, inhibiting the respiration of both type of leaves, its action cannot be used as evidence for or against the participation of polyphenoloxidase in the respiration of the injected leaf pieces.

CONCLUSIONS

It seems probable that the stimulating effect of catechol on ivy leaf respiration involves polyphenoloxidase. The evidence for this is threefold. Other dihydroxyphenols which act as substrates for polyphenoloxidase *in vitro* have a similar effect to that of catechol. Catechol has an effect in nitrogen negligible by comparison with its effect in air. The respiration of non-polyphenoloxidase-containing leaves is only slightly raised by catechol.

The effect of catechol on ivy leaf respiration is different from that previously described for potato by Boswell and Whiting (1938) and Baker and Nelson (1942) and for spinach by Bonner and Wildman (1946). These workers found that the oxygen uptake was at first stimulated, but later markedly inhibited; the carbon dioxide production showed the same initial stimulation in the case of potato; in spinach the rate declined as soon as the catechol was added. In ivy the stimulation of both oxygen uptake and carbon dioxide output is equal and decreased with time, but no inhibition is found.

The failure of catechol to cause inhibition in the ivy might be due to a more active system of dehydrogenases present in that tissue preventing the formation of the oxidation products which produce inhibition in other tissues and in *in vitro* experiments. The addition of catechol could be regarded as inducing the functioning of polyphenoloxidase in the cell and might be due to spatial separation of the enzyme from its carrier, especially if the polyphenoloxidase is located in the chloroplasts as shown for spinach (Arnon, 1949, and Li and Bonner, 1947). Lack of inhibition by cupferron suggests that polyphenoloxidase does not take part in normal respiration. Whether polyphenoloxidase is the normal oxidase in ivy leaf respiration or only an extra system, it seems clear that the extra oxygen uptake induced by adding its carrier is linked with utilization of a substrate similar if not identical with that used in normal respiration since the respiratory quotient remains unchanged even when there is a very considerable percentage increase in the rate of oxygen uptake.

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Studies with Radioactive Tracers in Plant Nutrition

III. THE EFFECT OF RADIATION FROM PHOSPHORUS-32 ON THE GROWTH AND PHOSPHATE UTILIZATION OF BARLEY SEEDLINGS

R. P. MARTIN AND R. SCOTT RUSSELL

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SUMMARY

The effect of radiation from P^{32} on the growth and phosphate absorption of barley seedlings was studied in water culture. Concentrations of carrier phosphate ranging from less than 0·01 to 31 p.p.m. P were employed for varying periods.

Between experiments wide variations occurred in the nature and extent of the effects of radiation. An increase in the proportion of the absorbed phosphate translocated to the shoots, and the reduction of root weight, were the most consistent symptoms. While severe injury was characterized by a reduction in both growth and phosphate absorption, intermediate levels of P^{32} on occasion caused increases in dry weight, in nutrient absorption, and in the development of lateral meristems.

The interrelation of the observed effects of radiation is discussed; the reduced metabolic activity of root meristems is considered to be the primary effect in the syndrome of radiation injury. Although injury is due to the accumulation of P^{32} in meristematic regions, no close correlation can be shown between its extent and the level of radiation received by the meristems. The inaccuracy of present methods for estimating the levels of radiation to which tissues are exposed has complicated the investigations of this subject. It appears, however, that the injurious level of radiation may vary from under 10 to over 40 rep. per day when the treatment period is 5 to 7 days. If active accumulation of phosphate occurs, the meristems of plant roots may receive this dosage when treated with more than 5 μ C. of P^{32} per litre for periods of 6 days.

The extent to which the effects of radiation may complicate the interpretation of the results of plant physiological investigations is considered.

INTRODUCTION

DURING preliminary experiments it was shown that significant injury could be induced by radiation from P^{32} when barley plants grown in water culture were supplied with 10μC. per litre of the external solution (Russell and Martin, 1949). Both growth and phosphate absorption were affected. Comparable evidence of injury was obtained when cereals were grown in soil (Russell, Martin, and Adams, 1950). It was apparent that injury was occasioned by the accumulation of phosphate in apical meristems, particularly root-tips.

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When conditions favoured rapid accumulation, the level of radiation to whic root-tips were exposed was several hundred times greater than at a point i the external medium. These results implied that radiation injury presente a considerably greater problem than had previously been supposed when P^3 is applied to intact plants, and that between experiments the pattern an extent of injury must be expected to depend on the extent of accumulation o phosphate and on its distribution within plants. Since the results of trace investigations could readily be invalidated if significant radiation injur occurred, it was clearly desirable to obtain further information and the wor described in this paper was therefore undertaken. A preliminary report o some aspects has already been published (Russell and Martin, 1952; Russell 1953).

The object of the investigation was limited to the determination of th types of effect on growth and phosphate utilization which could be induce in intact plants by activities of P^{32} in the range convenient for tracer work So that a representative picture of the symptoms of injury could be obtained the extent to which labelled phosphate was absorbed and the manner i which it was distributed within the plants was varied in different experiment by employing a wide range of concentrations of labelled phosphate in th external medium. The duration of treatment was also varied. Furthe differences in the absorption of P^{32} were brought about by the fact that th environment of the greenhouse in which the plants were grown was controlled to only a small extent; thus preliminary information could be obtaine on the response of plants to radiation from P^{32} under different condition It was realized that experiments of this type would be of limited value fo elucidating the mechanism of the biological action of ionizing radiation; tha question is beyond the scope of the present investigation.

EXPERIMENTAL

The cultural and analytical methods employed have been described elsewhere (Russell and Martin, 1953; Martin and Russell, 1950). 'Spratt Archer Barley, supplied by the National Institute of Agricultural Botany, was use throughout the work. Plants at the second-leaf stage, which had previousl been grown in the absence of external sources of phosphate, were supplied wit a balanced nutrient solution containing labelled phosphate. Ions other tha phosphate were used at the following concentrations in m.eq./l.: Mg^{++} 3; Ca^{++} 3 K^{+} 6; Na^{+} 2; NO_3^{-} 10; SO_4^{--} 4. In each experiment the concentration o labelled phosphate was kept at a constant level, and the treatments differe only in that varying activities of P^{32} were employed as tracer. In differen experiments concentrations of carrier used ranged from 31 to less than 0·0 p.p.m. P. Except when it is stated to the contrary, vessels containing 300 ml of solution were used, two plants being grown in each vessel. In some experiments the plants were transferred to tracer-free solutions after treatmen with P^{32}, so that their subsequent development could be studied. When samples were taken on more than one occasion those made immediately after treatment with P^{32} are termed Series I and the subsequent ones Series II.

In the assessment of the results, interest centres on the changes in growth and in the utilization of phosphate induced by P^{32}. The effects of P^{32} on growth are therefore presented as percentages of the values for treatments which received no P^{32}. The absorption of phosphate and its distribution between roots and shoots was determined by radioactivity assay; complications due to the phosphate already present in the plants were thus avoided. This procedure, however, made it necessary to use the plants supplied with the lowest activity of P^{32} in each experiment as the control for the assessment of effects on the utilization of phosphate. The level of P^{32} in this treatment was, it was judged, sufficiently low for significant radiation effects on phosphate metabolism to be unlikely. So that comparison can be made between different experiments, data for the control treatments of all experiments are summarized in Table I. The accumulation of P^{32} within the plants and the consequent pattern of injury was greatly affected by the concentration of carrier phosphate employed, and the experiments are therefore grouped according to the concentration of carrier.

A. *Concentration of labelled phosphate in culture solution less than 0·01 p.p.m. P*

Expt. 1. In this experiment (Russell and Martin, 1949) plants were treated with 0, 5, 10, 20, and 50 μC. P^{32}/litre in the presence of less than 0·01 p.p.m. P for 6 days in threefold replication. Effects on dry weight were then determined.

Expt. 2. The following activities of P^{32} were employed in the presence of 0·0017 p.p.m. P: 0, 0·6, 6, 60, and 100 μC./litre. Six replicates were sampled after 6 days (Series I); six further replicates were at the same time transferred to a balanced nutrient solution containing 10 p.p.m. P, but no P^{32}, and harvested after an interval of 14 days (Series II). Effects on growth and on phosphate absorption were examined on both occasions. In place of the standard 300-ml. culture vessels, containers holding 600 ml. of solution were employed.

The results of both experiments are summarized in Figs. 1 and 2. Similar trends in dry weight were induced by treatment with labelled phosphate for 6 days in both experiments. Root weight was reduced by increasing activities of P^{32}, but only in Expt. 1 was the effect significant at the end of treatment period with P^{32} (10μC./litre and higher treatments). However, the fact that a significant reduction was observed in Series II of Expt. 2 suggests that the decrease shown in Series I is indicative of a genuine effect. Shoot weight was increased in both experiments at an intermediate concentration of P^{32} (Expt. 1, 20 μC./l.; Expt. 2, Series I, 6 μ.C./l.).

In Expt. 2, Series II, striking effects on growth were shown; both shoot and root weight were reduced by 60 and 100 μC. P^{32}/l. Moreover, the

TABLE I

Data for control treatments in experiments on radiation injury.

Dry weights as mg./plant and phosphate contents as µg./plant

	Experiment No.	1	2	3	4	5	6	7
Period of treatment with labelled phosphate	Concentration of carrier phosphate (p.p.m. P)	0·01	0·0017	1	1	10	10	31
	Series I or Ia. Dry weight—total	92·6	57·3	152·7	65·8	97·7	91·0	111·6
	,, ,, root/shoot %	65·5	48·4	82·4	51·2	47·9	56·2	35·3
	Labelled phosphate content	—	0·305	133·1	64·4	1095	773	834
	,, ,, root/shoot %	—	306	83·7	120·7	30·1	49·1	40·2
	Series Ib. Dry weight—total	—	—	—	—	173·3	—	—
	,, ,, root/shoot %	—	—	—	—	45·9	—	—
	Labelled phosphate content	—	—	—	—	1490	—	—
	,, ,, root/shoot %	—	—	—	—	28·3	—	—
Subsequent period in tracer-free solution	Concentration of phosphate (p.p.m. P)	—	10	nil	31	10	nil	—
	Series II. Dry weight—total	—	190·1	241·1	119·3	480·8	229·0	—
	,, ,, root/shoot %	—	48·9	78·4	45·4	28·8	40·9	—
	Labelled phosphate root/shoot %	—	96·0	97·2	87·6	21·0	40·8	—
	No. of leaves on main axis	—	5	—	—	—	—	—
	Length leaf 3 (cm.)	—	14·6	—	—	—	—	—
	No. of emerged tillers	—	1·58	—	—	—	—	—

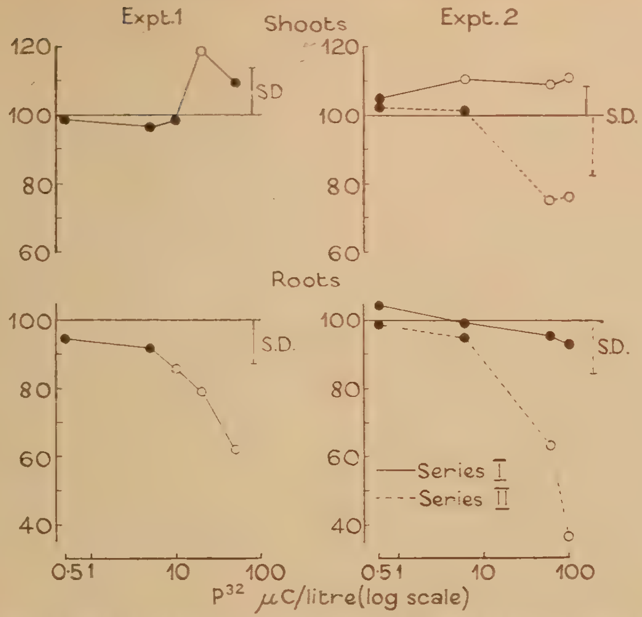

FIG. 1. Expts. 1 and 2. Less than 0·01 p.p.m. labelled P. Effect of radiation from P^{32} on the dry weight of barley plants. Values expressed as percentages of controls. Significant effects indicated by open circles.

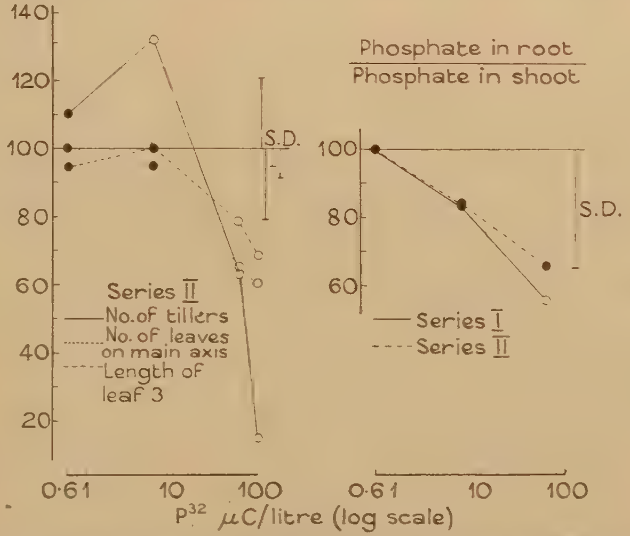

FIG. 2. Expt. 2. Less than 0·01 p.p.m. labelled P. Effect of radiation from P^{32} on the development of leaves and tillers, and on the distribution of absorbed phosphate in barley plants. Conventions as in Fig. 1.

number of leaves on the main axes of the plants was significantly decreased by these activities of P^{32} (Fig. 2). The number of tillers was increased by 6 μC. P^{32}/l. and reduced by higher activities.

The amount of labelled phosphate absorbed was not significantly affected in either series of Expt. 2. The ratio of labelled phosphorus in roots to that in shoots was, however, markedly reduced by radiation (Fig. 2).

B. *Concentration of labelled phosphate in culture solution 1·0 p.p.m. P*

Expt. 3. Plants were treated for 4 days with five levels of P^{32} forming a geometric series from 0·16 to 100 μC. l. In the control treatment no P^{32} was applied. Seven replicates were then sampled (Series I). Additional replicates which had received similar treatment with labelled phosphate were sampled after a subsequent period of 7 days in a phosphate-free medium (Series II).

Expt. 4. Plants were treated with 5 levels of P^{32} forming a geometric series from 0·36 to 45 μC. l., together with a control treatment without P^{32}. Six replicates were sampled after 5 days (Series I). Five additional replicates were sampled after further treatment for 7 days in a balanced nutrient medium containing 31 p.p.m. P (Series II).

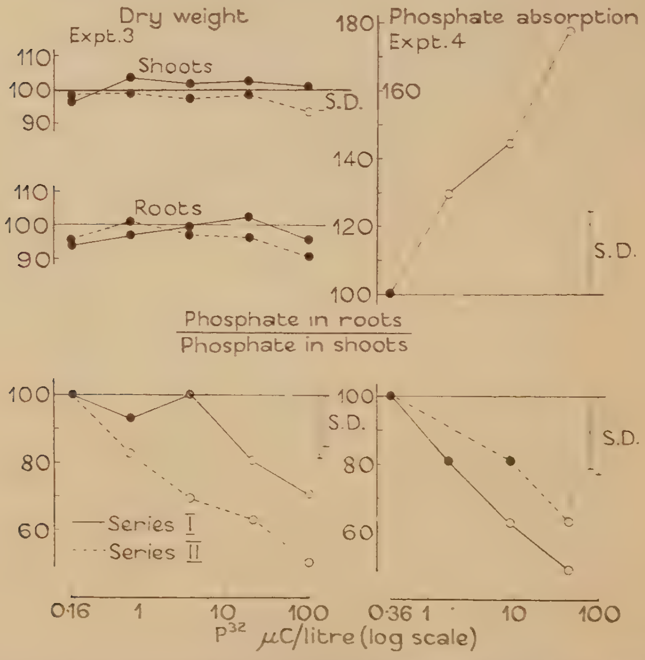

FIG. 3. Expts. 3 and 4. 1 p.p.m. labelled P. Effect of radiation from P^{32} on dry weight and on the absorption and distribution of phosphate in barley plants. Conventions as in Fig. 1.

No significant effects on dry weight were found at the end of the initial period in either experiment (Fig. 3). In Series II of Expt. 3 shoot weight was reduced to an extent which was just significant in the 100 μC. P^{32}/l. treatment (Fig. 3); no significant effects occurred in Expt. 4. Despite the absence of weight changes, the rate of initiation of leaves and their lengths was affected in a manner similar to that shown in Expt. 2. It would seem probable therefore that, had the experimental period been prolonged, a reduction in dry weight similar to that shown in Series II of Expt. 2 would have occurred.

In Expt. 3 the effects of radiation on the absorption of phosphate could not be examined since in all treatments at least 90 per cent. of the phosphate in the outer medium was absorbed. In Expt. 4, however, the plants were considerably smaller, and in the control treatment they absorbed less than 40 per cent. of the phosphate supplied; data on the effects of radiation on the absorption of phosphate could therefore be obtained (Fig. 3). Absorption was increased by radiation, the effect being significant in the 1·8 μC. P^{32}/l. treatment. In Expts. 3 and 4, as in Expt. 2, radiation markedly reduced the proportion of the absorbed phosphate which was found in the roots. The extent of this effect was, however, markedly different between the experiments. The lowest activities of P^{32} which caused a significant change in the ratio were: Expt. 3, Series I, 20 μC.; Series II, 0·8 μC.; Expt. 4, Series I, 9 μC.; Series II, 45 μC. These changes in the distribution of phosphate were not associated with comparable alterations in the distribution of dry matter between roots and shoots.

C. *Concentration of labelled phosphate in culture solution 10 p.p.m. P*

Expt. 5. Five levels of P^{32} were employed, forming a geometric series from 0·16 to 100 μC./l., together with a control treatment in which no P^{32} was employed. Four replicates were harvested after each of the following periods: 3 days in labelled phosphate (Series I*a*); 7 days in labelled phosphate (Series I*b*); 7 days in labelled phosphate followed by 7 days in a balanced nutrient solution containing 10 p.p.m. P (Series II).

Expt. 6. Five levels of P^{32} were employed forming a geometric series from 0·16 to 100 μC./l. Five replicates were harvested after treatment with labelled phosphate for 6 days (Series I). Five further replicates which had been given the same initial treatment with labelled phosphate were transferred for 6 days to a phosphate-free solution and then harvested (Series II). No treatment without P^{32} was used; the 0·16 μC. treatment therefore served as a control for the assessment of changes in both growth and phosphate absorption.

The only significant effects on the weight of roots at the end of treatment with labelled phosphate (Expt. 5, Series I*a* and I*b*; Expt. 6, Series I) was a reduction in the 4 μC. treatment of Expt. 5, Series I*a* (Fig. 4). Significant effects were, however, shown by plants which had been transferred to tracer free solutions. In Expt. 5, Series II, root weight was significantly increase by radiation in the 0·16 μC. treatment; no such effect occurred in Expt. 6 Series II. Shoot weight was increased in the 4 μC. treatment of Expt. 6

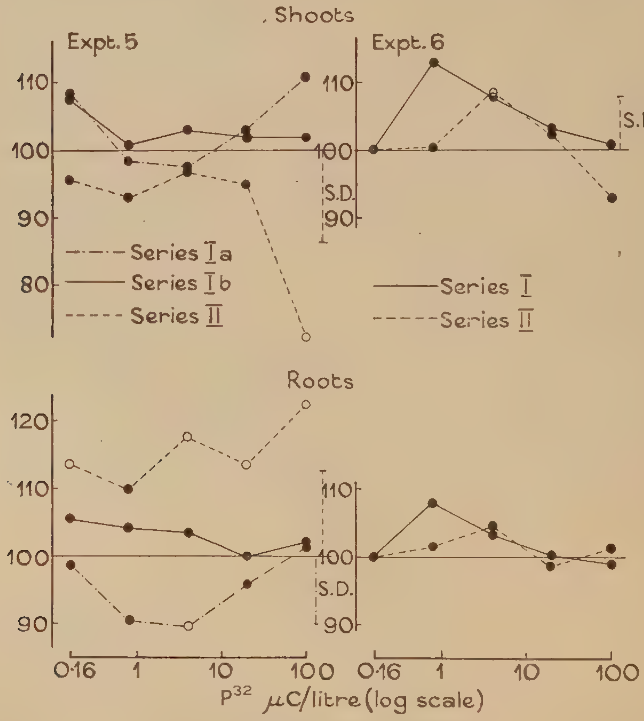

FIG. 4. Expts. 5 and 6. 10 p.p.m. labelled P. Effect of radiation from P^{32} on the dry weigh of barley plants. Conventions as in Fig. 1.

Series II, and depressed to an extent which fell just short of statistical significance in the 100 μC. treatment; in Expt. 5, Series II, shoot weight wa markedly depressed in the 100 μC. treatment.

The amount of phosphate absorbed was not significantly altered by radiation except in the 4 μC. treatment of Expt. 5, Series I. In both experiments however, the labelled phosphate content of roots relative to that of shoots wa decreased as in the experiments previously described. This effect was more marked at the first sampling occasion in each experiment than on late occasions.

The effects resulting from 4 μC./l. in Expt. 5 deserve special notice. I Series I*a* this activity of P^{32} induced a greater reduction in root growth than the higher activities (Fig. 4), while in Series I*b* and II it showed the lowest ratio of phosphate in roots to that in shoots (Fig. 5). Although the magnitude of the effect was considerable only in Series I*b*, these three results taken together are indicative that under certain circumstances increasing activities of P^{32} may reduce the apparent effect of radiation on certain physiological processes.

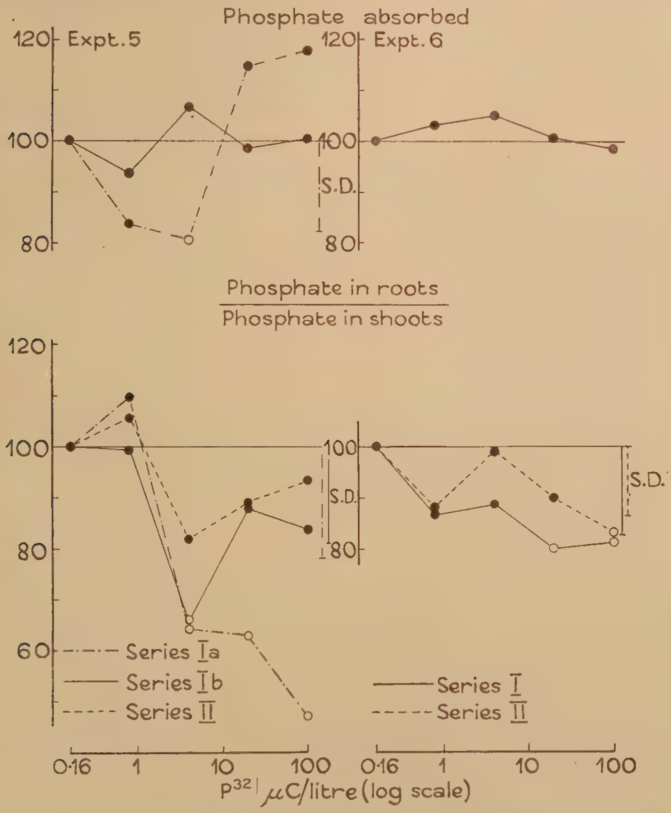

FIG. 5. Expts. 5 and 6. 10 p.p.m. labelled P. Effect of radiation from P^{32} on the absorption and distribution of phosphate in barley plants. Conventions as in Fig. 1.

D. *Concentration of labelled phosphate 31 p.p.m P*

Expt. 7. This experiment (Russell and Martin, 1949) was carried out simultaneously with Expt. 1 and with plants from the same stock. Plants in threefold replication were treated with 0, 0·5, 5, 10, 20, and 50 µC. P^{32}/l. for 6 days. Phosphate content was not determined by tracer assay in the 0·5 µC. treatment; thus the 5 µC. treatment served as the control for the assessment of effects on the absorption of phosphate in plants supplied with higher activities.

The dry weights of both roots and shoots were significantly reduced in the 50 μC. treatment (Fig. 6) and the phosphate content of roots was significantly reduced in the 10 μC. and higher treatments. No significant effects on the phosphate content of shoots was, however, observed. A tendency for the ratio of phosphate in roots to that in shoots to be depressed by radiation was apparent, but it fell short of the level of statistical significance.

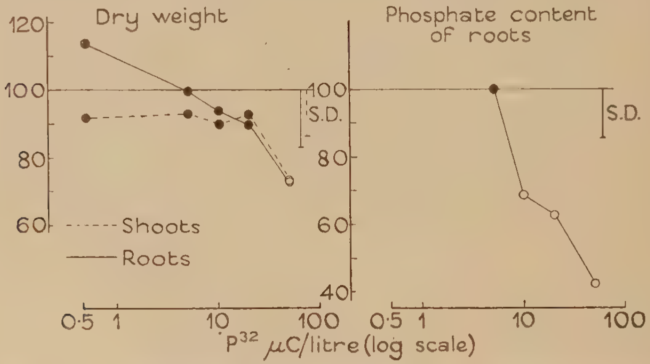

FIG. 6. Expt. 7. 31 p.p.m. labelled P. Effect of radiation from P^{32} on dry weight and on the absorption of phosphate by barley plants. Conventions as in Fig. 1.

THE SYNDROME OF RADIATION INJURY

The experiments here described show that the injury induced in plants by absorbed P^{32} is complex and highly variable: depending on conditions, growth, and phosphate absorption may be accelerated or retarded. It appears that the primary injury occurs in meristematic tissues, especially those of the root. Cells in these zones are more sensitive to injury than mature tissues (Gray and Scholes, 1951), and the metabolic accumulation of phosphate may cause them to be exposed to much higher levels of radiation (Russell and Martin, 1949). The morphological effects observed in the present experiments are indicative of abnormalities in meristematic activity, and the histological studies of Mackie, Blume, and Hagen (1952) provide direct evidence on this point. Thus the effects on the utilization of phosphate and on growth observed in these investigations are to be regarded as consequences of prior injury to meristematic tissues.

The most consistent symptom of injury and often the earliest to appear was an increase in the proportion of the absorbed phosphate found in shoots. In five of the six experiments in which the distribution of phosphate was examined this effect was statistically significant; in the sixth (Expt. 7) a tendency in the same direction was shown. The results of Blume, Hagen, and Mackie (1950) show the same trend, which was, however, not discussed by these authors (Table II). A similar effect on the partition of absorbed phosphate between roots and shoots has been caused by enzymatic inhibitors under certain circumstances (Russell, Martin, and Bishop, 1953). It is considered that the mode of action of radiation may be similar to that of the inhibitors, namely that the metabolic utilization of phosphate in root growth is reduced to a greater extent than the mechanism responsible for the transference of phosphate to the stele, and its subsequent movement to the shoot.

A further similarity between the effects of inhibitors (Russell *et al.*, loc. cit.) and those of radiation is that the absorption of phosphate is under some circumstances increased. In the experiments with inhibitors this occurred when large proportions of the absorbed phosphate would, in untreated plants, have been retained in metabolic systems in the roots. It appeared that a significant proportion of phosphate retained in this manner was subsequently lost to the outer medium, and it was considered that the apparent increase in absorption occasioned by inhibitors was due to the prevention of such losses. Possibly, therefore, the increased absorption of phosphate observed in Expt. 4 may be similarly explained.

TABLE II

Effects of different levels of P^{32} *in a* 10^{-3} *molar phosphate nutrient solution upon phosphorus content of barley plants* (*Blume, Hagen, and Mackie, 1950*)

Activity of P^{32} μC./l. (1)	Phosphorus content Root % (2)	Shoot % (3)	Root/Shoot (4)
0	1·32	1·30	1·02
4	1·28	1·26	1·02
8	1·20	1·31	0·92
16	1·23	1·37	0·90
32	1·21	1·40	0·86
64	1·21	1·33	0·91
128	1·29	1·46	0·88
Standard error	0·029	0·049	—

Note: Columns 1, 2, and 3 are taken from Table 4 of Blume, Hagen, and Mackie, from which column 4 has been calculated.

The effects of the initial reduction of meristematic activity on the subsequent development of the plant may now be considered. Clearly, if the reduction of meristematic activity is sufficiently great, root growth and phosphate absorption will in due course decrease, as was observed in Expt. 1, Expt. 2, Series II; Expt. 5, Series I*a*, and Expt. 7. No effect on root growth was, however, shown in Expts. 3, 4, and 6; it may be assumed therefore that the initial injury, though sufficient to affect the distribution of phosphate, was not great enough to cause an alteration in the dry weight of roots. A contrasting situation was apparent in Expt. 5, Series II; root weight was there increased. This effect, which developed only after the plants had been transferred to tracer-free solutions, may have resulted from the stimulation of lateral meristems following the initial injury to the apical meristems. This interpretation is supported by current work in this laboratory which shows that the elongation of unirradiated lateral roots is stimulated when root-tips are irradiated with 300 r. of X-radiation (Boyd, unpublished). Furthermore, evidence of root regeneration has been obtained in soil (Russell, Martin, and Adams, 1950) and a comparable stimulation in the branching of shoots was observed in the present investigation (Expt. 2, Series II). Evidence that growth is accelerated under certain circumstances is also provided by the results of Dion *et al.* (1949); Bould, Nicholas, and Thomas (1950), and Blume (1951).

The reduced metabolic activity in the roots will, however, also influence the metabolism of the shoots, and further effects on root development may in turn be caused by subsequent injury to the shoots. If the primary radiation injury is confined to the roots, the consequent reduction of carbohydrate utilization therein may be expected to increase shoot weight. This situation was observed after treatment with intermediate activities of P^{32} in Expts. 1 and 2, Series I. In both experiments low concentrations of labelled phosphate were employed, and only a small fraction of the absorbed phosphate was translocated to the shoot. Exposure to higher levels of radiation will, however, be likely to cause injury to the apical meristems of shoots which will in turn cause a reduction in the size and number of leaves produced on the main axis. As with roots, the ultimate size of the shoots will depend on the severity of injury. Thus, in Expt. 2, Series II, the highest levels of radiation (60 and 100μC. l). led to a marked reduction in shoot weight, whereas 6μC. l. caused a significant increase in tillering. This effect on tillering was not associated with a significant effect on dry weight; it is, however, reasonable to infer that in a more prolonged experiment increased tillering would have been reflected in greater dry weight The increased shoot weight observed in Expt. 6, Series II, was perhaps due to this cause. The mechanism by which radiation injury to plant meristems, whether roots or shoots, causes an increase in the activity of other meristems cannot at present be suggested. The simplest explanation is that the reduced utilization of metabolites in the primary meristems is the operative factor. However, the observed effects appear compatible with the hypothesis that the effects on the synthesis or distribution of auxin or other growth-controlling substances may be important. Skoog (1935), Smith and Kersten (1942), and Gordon and Weber (1952) have shown that the production of auxin is readily inhibited by radiation.

An examination of the relationship between the activity of P^{32} applied and the extent to which different symptoms were developed sheds further light on their interrelationships. The effects of different activities of P^{32} on the ratio of phosphate in roots to that in shoots, on the depression of root weight, and on the increase in shoot weight are shown in Figs. 7, 8, and 9 respectively for the experiments in which the greatest effects were induced. The root to shoot phosphate ratio was depressed steeply when the activity of P^{32} was raised from zero to 5–10 μC. l.; thereafter the depression was less rapid (Fig. 7). Thus the relationship was approximately logarithmic. The depres-

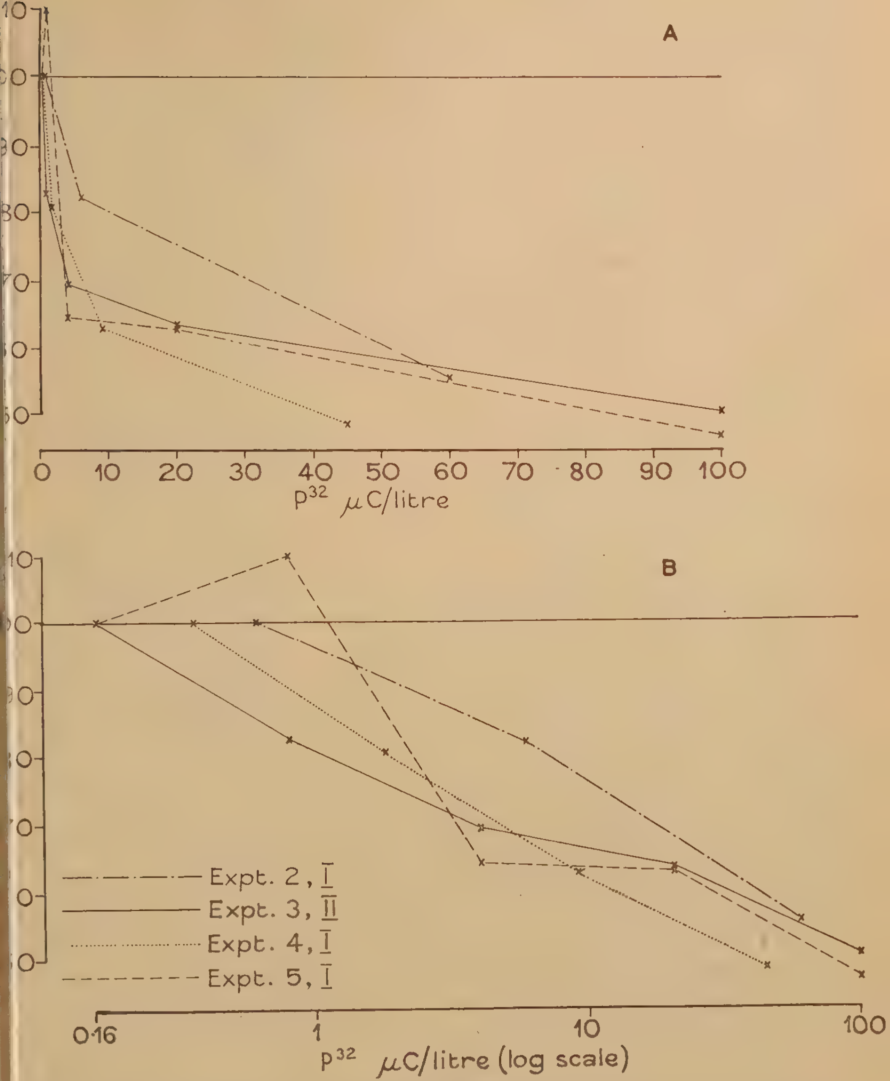

FIG. 7. Relationship between activity of P^{32} in culture solution and ratio of phosphate in roots to that in shoots in experiments in which the greatest reduction occurred. Values expressed as percentages of controls. A, activity of P^{32} linear scale; B, activity of P^{32} logarithmic scale.

sion of root weight bore, however, a linear relationship to activity of P^{32} (Fig. 8). Information is insufficient to justify a discussion of the difference in the form of these curves. Both, however, contrast sharply with the complex

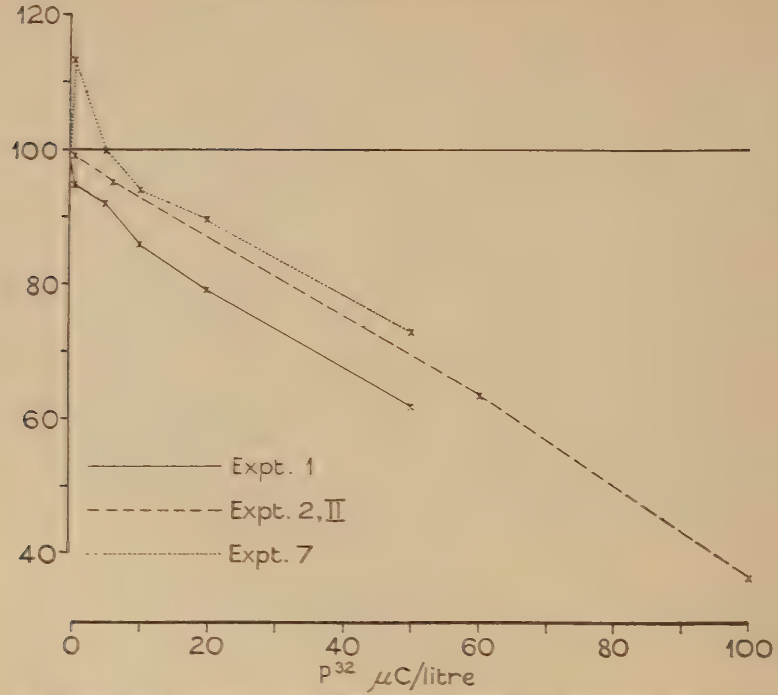

FIG. 8. Relationship between activity of P^{32} in culture solution and root weight in experiments in which the greatest reduction occurred. Values expressed as percentages of control.

relationship between the activity of P^{32} and the increase in shoot weight shown in Fig. 9. In two of the three experiments for which results are represented in this figure the maximum effect was induced by intermediate activities of P^{32}. Moreover, a similar situation occurred with regard to the stimulation of tiller initiation in Expt. 2 (Fig. 2). These relationships suggest that while the reduction of the metabolic activity of roots and the consequent changes in the distribution of phosphate are the primary physiological effects, the stimulations of growth which may be induced by radiation are indirect effects dependent on the interaction between metabolic processes which have been interfered with to varying extents. It cannot, however, be assumed that the reduction of the metabolic activity of root meristems is due to the action of radiation on a single metabolic system. When only mild symptoms of injury occurred, the magnitude of this effect sometimes *decreased* when the activity of P^{32} was raised from intermediate to higher levels. This was shown with respect to both root weight and phosphate absorption in Expt. 5, Series I *a* (Figs. 4 and 5). This suggests that injury is the result of interference with at least two mechanisms which are sensitive to radiation to differing extents, and which are related in such a manner that the partial inhibition of the more resistant mechanism lessens the overall effect on growth of interference with the more susceptible mechanism. Such a situation would not be surprising in the light of current knowledge of the indirect effect of ionizing radiation in biochemical systems (cf. Gray, 1952).

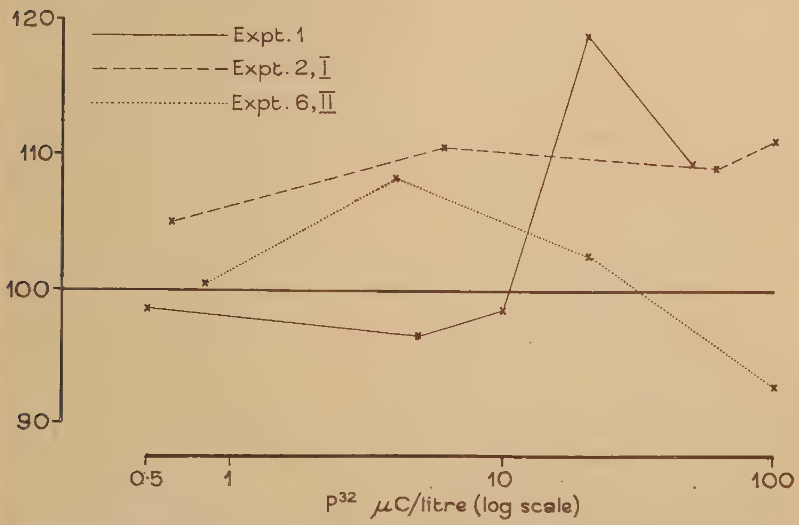

FIG. 9. Relationship between activity of P^{32} in culture solution and shoot weight in experiments in which the greatest increase occurred. Values expressed as percentages of controls.

CAUSES OF VARIATION IN THE EXTENT OF INJURY INDUCED BY LABELLED PHOSPHATE

Between experiments considerable differences occurred in the levels of P^{32} which induced a significant degree of injury. Comparable variation was shown between experiments in a parallel investigation with plants grown in soil (Russell, Adams, and Martin, 1949; Russell, Martin, and Adams, 1950). Russell (1953) has reviewed the results of other investigators which show similar variability (e.g. Blume, Hagen, and Mackie, 1950; Bould, Nicholas, and Thomas, 1951). As differences in accumulation of P^{32} seem to be a likely cause of such effects, so the relation between P^{32} content of plants and the extent of injury was examined. In Table III, columns 1 and 2 show respectively the highest external concentration of P^{32} which caused no significant injury in each experiment, and the resultant root content. In columns 3 and 4 similar data are given for the lowest external concentration which reduced either the amount of absorbed phosphate retained in the roots or in root dry weight. It is clear that although there is a general relationship between the concentration of P^{32} in the tissues and the extent of injury, differences

TABLE III

Comparison of concentrations of P^{32} *in plants in relation to symptoms of radiation injury in different experiments*

Criteria of injury used were reduction of dry matter content of root or shoot, alteration of phosphate absorption, or alteration of root shoot phosphate ratio.

		Highest concn. of P^{32} which caused no significant injury		Lowest concn. of P^{32} which caused significant injury		Specific activity of P^{32}	
Concentration of carrier p.p.m. P	Expt. No.	Extn. soln. μC. P^{32}/l. (1)	Plant roots μC. P^{32}/g. dry weight (2)	Extn. Soln. μC. P^{32}/l. (3)	Plants roots μC. P^{32}/g. dry weight (4)	Highest treatment without injury (5)	Lowest treatment causing injury (6)
$>$ 0·01	1	5·0	10·9	10	26·0	$>1\times10^5$	$>1\times10^5$
	2, I	0·6	2·79	6	27·1	$3{\cdot}5\times10^5$	$3{\cdot}5\times10^6$
1·0	3, I	4·0	3·49	20	15·0	$4{\cdot}0\times10^3$	$2{\cdot}0\times10^4$
	4, I	1·8	2·95	9	15·6	$1{\cdot}8\times10^3$	$9{\cdot}0\times10^3$
10	5, I*a*	0·8	0·64	4	2·08	$8{\cdot}0\times10^1$	$4{\cdot}0\times10^2$
	5, I*b*	0·8	0·45	4	1·83	$8{\cdot}0\times10^1$	$4{\cdot}0\times10^2$
	6, I	4	3·2	20	14·6	$4{\cdot}0\times10^2$	$2{\cdot}0\times10^3$
31	7	5	1·70	10	2·46	$1{\cdot}6\times10^2$	$3{\cdot}2\times10^2$

between experiments cannot be attributed solely to differences in the mean concentration of P^{32} in the tissues.

Since root-tips are exposed to particularly high levels of radiation from accumulated P^{32} (Russell and Martin, 1949), it seemed possible that the level of radiation to which the root-tips were exposed would be more closely correlated with the extent of injury than the mean concentration of P^{32} in the root system. This was examined in four of the experiments already described (Table IV). The calculation of radiation dosage is complicated both by the uneven distribution of P^{32} and because the thinness of the roots (0·2–0·6 mm. mean diameter) caused a considerable fraction of the radiation from the P^{32}

TABLE IV

Estimate of the levels of radiation in root-tips and in the culture solution in different experiments. Treatments showing no significant radiation effect are in parentheses

	Roentgens-equivalent-physical/day				
	Root-tips				Initial culture solution
Treatment μC. P^{32}/l.	Experiment No. 1	3	5	7	
4	—	8·5	2·8	—	0·16
5	(41)	—	—	(1·8)	0·2
10	87	—	—	3·4	0·4
20	86	35	12	9·1	0·8
50	93	—	—	7·3	2·1
100	—	165	52	—	4·2

within them to escape from the tissues. Thus the roots were exposed to only a small fraction of the radiation dose which the same concentration of P^{32} would cause in an object of infinite size. In a previous paper (Russell and Martin, 1949) an endeavour was made to overcome this difficulty by assuming that the apical 5 mm. of a root receives the same dose of radiation as the centre of a sphere of equal volume containing the same concentration of P^{32}. More recently Hammersley (1953) has provided a method for determining the proportion of the radiation which is retained in small cylinders and the present calculations have been made by this method. The dosages thus determined are approximately one-third of those stated by Russell and Martin (1949). The use of the Hammersley method underestimates radiation dosages, since it is necessary to assume that the P^{32} is evenly distributed through the meristems, and it has been shown by radio-autographs that this is not so. On the other hand, the earlier calculations may well have led to large errors in the opposite direction, since considerably more radiation will be absorbed in a small sphere than in a cylinder of the same volume. Despite these limitations the data show no close correlation between the radiation dose and the extent of injury. An estimated dose of less than 10 rep./day to the roots caused effects on growth or phosphate utilization in Expts. 3 and 7; by contrast, in Expt. 1 at least four times that dosage had no significant effect. However, it seems safe to conclude that a dose in the order of 50–100 rep./day will induce significant injury under all circumstances.

Many investigators have considered that injury in such studies is particularly related to the specific activity of P^{32} employed. The specific activity for the lowest treatments in which injury was induced and the highest treatments in which it did not occur in the present investigation were therefore compared (Table III, cols. 5 and 6). It is evident that there was little correlation between the specific activity of P^{32} and the occurrence of injury.

It is concluded therefore, from the data presented in Tables III and IV, that the variable sensitivity of plants to injury in the present experiments cannot be explained solely by differences in the accumulation of P^{32} within the tissues, or in its external concentration, important though these effects are. Quastler and Baer (1948) observed that the extent of injury induced by a given level of radiation varied with the rate of growth of the experimental material, and the work of Barron and Dickman *et al.* (1949), and Hollaender, Stapleton, and Martin (1951) indicates that metabolic differences can cause similar effects. It therefore seems probable that physiological differences between plants at the beginning of the experiments were an important cause of variation in their sensitivity to radiation.

CONCLUSIONS

The foregoing discussion shows that the pattern of injury induced in intact plants by radiation from absorbed radioactive isotopes is susceptible to no simple analysis. Thus at the present time the assessment of the extent of injury which will be induced under a given set of conditions must rest primarily on the results of *ad hoc* experiments. Certain inferences with regard to the use of P^{32} as a tracer in experiments with intact plants may, however, be drawn. It appears that no lower limit of activity can be defined below which radiation from accumulated isotopes will be entirely without physiological effects. In the present work 0·15 μC. P^{32}/l. did on one occasion induce significant injury; in another 0·8 μC. P^{32}/l. was injurious. These treatments caused the meristematic tissues to receive between 5 and 10 rep. per day. It would seem unlikely, however, that in a series of investigations such low levels of radiation would induce consistent effects; thus the precision rather than the validity of the experimental results will be affected. If, on the other hand, the meristematic tissues of plants receive a total radiation dose of the order of 50–100 rep. per day, a significant effect of radiation is likely. Levels of radiation in this order may well arise if activities in excess of 5 μC. P^{32}/l. of culture solution are applied to actively growing plants for 6 days or more. Since the use of lower activities may be inconvenient in experiments lasting for more than a few days, the possibility cannot be dismissed that radiation injury may be a limiting factor in experimental design. Under such circumstances the results of physiological investigations are necessarily of doubtful validity unless it has been established that the effects of radiation in the processes under investigation are insignificant by comparison with treatment effects. Because of the wide variability in the pattern of injury, no cursory examination of plants treated with different activities of tracer can ensure that this requirement is fulfilled. As the present results show, the absorption and distribution of phosphate may be affected without observable effects on growth, while the pattern of growth as between apical and lateral meristems may be greatly modified although in size and in appearance the plants appear normal.

The consequence of these limitations and the appropriate method whereby they can be circumvented will necessarily depend on the nature of each investigation.

ACKNOWLEDGEMENTS

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Metal Interrelationships in Plant Nutrition

2. THE RELATION OF METAL TOXICITY, MOLYBDENUM AND NITROGEN SOURCE TO CHLOROPHYLL AND MAGNESIUM CONTENT OF BEET IN SAND CULTURE

E. J. HEWITT

Agricultural Research Council Unit of Plant Nutrition (Micro-nutrient elements),
Long Ashton Research Station, University of Bristol

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SUMMARY

Sugar beet and spinach beet were grown in sand culture with different sources of nitrogen or iron and different levels of molybdenum and heavy metals. Chlorophyll and some magnesium fractions were determined.

Extra molybdenum accentuated chlorosis caused by excess of Mn^{++}, Cr^{+++}, Zn^{++}, and Cu^{++} and decreased it in the presence of CrO_4^{--} with nitrate.

In the presence of these ions urea reduced chlorophyll in young and increased it in old leaves of sugar beet. Effects of urea and molybdenum were additive and independent. Ammonium sulphate caused increased chlorosis of spinach beet with Mn^{++} and Zn^{++}.

The existence of an acetone-soluble magnesium fraction other than chlorophyll was shown. Excess Cu^{++} and Zn^{++} decreased total magnesium; Mn^{++} did not. Mn^{++} and Zn^{++} reduced chlorophyll, increased the non-chlorophyll acetone-soluble magnesium, but reduced other fractions. Ferrous iron increased total magnesium content and also increased the acetone-soluble fractions in the presence of Mn^{++} and Zn^{++}. Extra molybdenum decreased the acetone-soluble magnesium fractions but did not affect the total magnesium content.

Significant interactions between Mo, N, Fe, and heavy metals were also disclosed.

THE ability of certain 'heavy' metals to induce iron deficiency and other effects in various crops was described by Hewitt (1948, 1953). Millikan (1947) concluded that extra molybdenum mitigated the iron-deficiency chlorosis and the toxic effects produced by several heavy metals given to flax in water cultures, but he did not make quantitative determinations of the pigments present. The work described here was begun in 1948 to obtain quantitative estimates of the extent to which chlorophyll production was affected by the various 'heavy' metal and molybdenum combinations. Special attention was given to the effect of nitrogen supply as the molybdenum effect was thought possibly to concern some aspect of nitrogen metabolism. The work was later extended to include examination of some magnesium fractions in the hope that this would help to elucidate the factors limiting chlorophyll production. A preliminary account was given by Hewitt (1951).

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EXPERIMENTAL

Methods. The sand-culture methods were those described previously (Hewitt, 1953). Nitrogen was given at 8 mM./l. as nitrate or urea in 1949 and as nitrate or ammonium sulphate in 1950. Molybdenum was given at 0·0012 m.eq./l.Mo^{VI} (Mo_1) and at 0·6 m.eq./l. (Mo_2) in 1948 or 0·3 m.eq./l. (Mo_2) in 1949 and 1950. Iron was given as ferric citrate at 0·075 m.eq./l. in 1949 and at 0·075 m.eq./l. as Fe^{+++} or at 0·05 m.eq./l. Fe^{++} as $FeSO_4$, to give the same concentration of iron, in 1950.

The excess heavy metal treatments were in all instances superimposed on those already present in the basal solutions where the normal levels (Mn 0·02, Cu and Zn 0·002 m.eq./l.) were given. The excess concentrations were given at 0·5 m.eq./l. Cr^{+++}, CrO_4^{--}, Mn^{++}, Co^{++}, Cu^{++}, Zn^{++}, or Cd^{++} to sugar beet, and at 0·6 m.eq./l. Mn^{++}, Cu^{++}, or Zn^{++} to spinach beet.

In the sugar-beet experiment the basal series of treatments comprised the 4 factorial combinations of 2 sources of nitrogen and 2 levels of molybdenum. These combined with 7 excess metal treatments and the common basal level provided a 32-treatment factorial. A split plot layout with respect to nitrogen sources was used. In the spinach-beet experiment the basal series comprised 8 combinations, as 2 valencies of iron made an additional 2 factors. These 8 basal treatments were tested with the 3 metals each separately in excess or at their common basal concentration, thus giving 32-treatment combinations. The excess metal factors and common basal level were confounded with sub-blocks in each replicate as their effects were expected to be great. There were 12 plants in each replicated treatment.

The sugar beet were sampled in July, August, and October for total chlorophyll. The samples, which weighed about 1 g., comprised small areas taken at random from young or old leaves from several plants. They were placed in a refrigerator and weighed accurately as soon as possible afterwards. They were frozen with solid CO_2, mixed with approximately 50 mg. $CaCO_3$, and homogenized in a small pyrex glass macerator with emery powder and 85 per cent. acetone. The extracts were filtered in darkness. The colourless residues were washed with 85 per cent. acetone and the volume made up to 50 ml. The solutions were stored in a refrigerator and the chlorophyll was measured the same day with a Spekker absorptiometer. A calibration curve was prepared from a chlorophyll solution whose concentration was determined from transmittencies measured at the National Physical Laboratory according to the formula and methods given by Comar (1942), Comar and Zscheile (1942), Comar, Benne, and Buteyn (1943).

In 1950 chlorophyll was extracted from spinach beet by thrice homogenizing 10-g. samples of all foliage, except necrotic or damaged material, in 85 per cent. acetone in a Townson and Mercer top-drive macerator and the volume made up to 250 ml. after filtration.

Magnesium in the acetone extracts and in the original samples was determined on the spinach-beet material after nitric-perchloric acid digestion using the method given by Hunter (1950). Magnesium in chlorophyll itself was found by calculation from the chlorophyll contents. The non-chlorophyll acetone-soluble fraction was found by difference between that calculated for chlorophyll, and the total contents in the acetone extracts.

EXPERIMENTAL RESULTS

Quantitative data on total chlorophyll and on yields and dry weight have been deposited at the British Museum. Results are given here in a condensed form to show the main trends. Table I *a* and *b* refers to sugar beet and Table II refers to spinach beet. The following notes explain the methods used to present the results.

In Table I *a* the effects of excess levels of the heavy metals on sugar beet are compared with each other or with a basal level containing the normal levels of micronutrients and such traces of other metals as were provided by impurities in the culture solutions. For conciseness and statistical purposes the effects of the nitrogen source are given as means for the normal and high molybdenum levels, and effects of molybdenum are based on the means of both sources of nitrogen. Interaction tables for molybdenum and nitrogen shown in Table I *b* are presented for means of all heavy metal treatments.

TABLE I *a*

Quantity of chlorophyll (mg./100 g. fresh wt.) in sugar-beet leaves following culture in presence of heavy metals. Samples taken in July

Differences exceeding 2 and 3 times the S.E. are significant at approximately 5 and 1 per cent. respectively.

	Treatment		Basal levels	Excess levels							Mean
				CrO_4	Cr	Mn	Co	Cu	Zn	Cd	
Young leaves	NO_3	at both Mo levels	154·6	103·7	120·5	95·8	8·9	19·8	75·2	10·0	73·6
	Urea		105·0	15·8	58·8	48·8	9·1	12·4	64·1	4·0	39·8
	Mo_1	irrespective of N treatment	134·2	44·2	115·1	108·2	8·7	20·1	98·9	4·9	66·8
	Mo_2		125·4	75·4	64·2	36·4	9·3	12·1	40·4	9·1	46·5
	Mean		129·8	59·8	89·6	72·3	9·0	16·1	69·6	7·0	56·7

S.E. means of horizontal columns ±3·20 S.E. horizontal comparisons ±11·09
S.E. means of vertical columns ±4·53 S.E. vertical comparisons ±9·05

Degrees of freedom 22

Old leaves	NO_3	at both Mo levels	87·6	64·6	72·4	71·6	75·6	81·9	58·0	42·6	69·3
	Urea		130·9	73·4	104·6	89·7	67·5	93·2	91·2	58·6	88·6
	Mo_1	irrespective of N treatment	106·7	63·6	94·8	89·0	71·3	82·0	89·4	47·1	80·5
	Mo_2		111·8	74·4	82·3	72·2	71·8	93·1	59·8	54·2	77·4
	Mean		109·2	69·0	88·5	80·6	71·6	87·6	74·6	50·6	79·0

S.E. means of horizontal columns ±2·43 S.E. horizontal comparisons ±9·53
S.E. means of vertical columns ±4·68 S.E. vertical comparisons ±6·86

Degrees of freedom 22

In Table II only the significant treatment effects on magnesium fractions are shown. The comparisons are arranged to show whether increases or decreases occurred in the respective fractions as the result of changes in heavy metal status, qualified where necessary according to the specific conditions of iron valency, molybdenum level, and nitrogen supply. The effects of comparisons of the paired variables for iron, molybdenum, and nitrogen are also given, qualified according to the heavy metal status and the other nutrient treatment factors. The estimates of significance are based on the factorial analysis of the data, and thus, for example, an effect for iron found in the presence of NO_3 implies that values for both levels of molybdenum were taken into consideration. The existence of numerous large interactions between N, Fe, and Mo has therefore resulted in the neutralization of a number of effects which would otherwise probably achieve significance. As far as possible the main interactions have been shown in association with the treatment effects to which they are related.

TABLE I*b*

Mean chlorophyll contents for all excess heavy metal and basal treatments

	Young leaves			Old leaves		
	NO_3	Urea	Mean	NO_3	Urea	Mean
Mo_1 . .	82·5	57·0	66·8	69·4	91·5	80·4
Mo_2 . .	64·6	28·5	46·5	69·2	85·7	77·4
Mean . .	73·6	39·8	56·7	69·3	88·6	79·0
S.E. for means . .			±3·20			±2·43
S.E. for vertical and horizontal comparisons . .			±4·53			±3·43
S.E. for diagonal comparisons			±5·54			±4·77

Yields of sugar beet

Shortage of space precluded the presentation of yield data here. Briefly, yields were progressively depressed in the following order ($p = 0{\cdot}01$):

NO_3 series: basal > excess Cr^{+++}, $Mn^{++} > Zn^{++}$, $CrO_4^{--} > Cu^{++}$, $Cd^{++} > Co^{++}$.

Urea series: basal > excess Zn^{++}, Cr^{+++}, $Mn^{++} > CrO_4^{--}$, Cu^{++}, Cd^{++}, Co^{++}; also $CrO_4^{--} > Cd^{++}$, Co^{++}.

Mo_1 series: basal > excess Mn^{++}, Zn^{++}, $Cr^{+++} > CrO_4^{--} > Cu^{++} > Cd^{++} > Co^{++}$.

Mo_2 series: basal > excess Cr^{+++}, CrO_4^{--}, Mn^{++}, $Zn^{++} > Cu^{++}$, Cd^{++}, Co^{++}.

NO_3 > Urea $p = 0{\cdot}01$ all treatments except excess Co^{++} and Cu^{++}.

$Mo_1 > Mo_2$ $p = 0{\cdot}01$ all treatments except basal, excess CrO_4^{--}, Co^{++}, Cd^{++}.

Dry weight

Percentage dry weight in foliage of spinach beet was often increased ($p = 0{\cdot}05$), by excess zinc, by extra molybdenum, ferrous iron, or ammonium sulphate; a similar increase occurred in sugar beet given excess cobalt or zinc.

Chlorophyll content

The behaviour of spinach beet qualitatively resembled that of sugar beet, Table I *a* (see Hewitt, 1953), but the former showed relatively greater tolerance to the metal excesses.

The effects of extra molybdenum were striking and consistent in each experiment. Extra molybdenum greatly accentuated chlorosis in the presence of excess Cr^{+++}, Mn^{++}, Cu^{++}; and Zn^{++} given to sugar beet, and in the presence of Mn^{++}, Cu^{++}, and Zn^{++} given to spinach beet in the presence of nitrate. No interaction could be detected with Co^{++} or Cd^{++}, where chlorosis was severe at both molybdenum levels. In the presence of CrO_4^{--} extra molybdenum *decreased* chlorosis in sugar beet. These effects were clearly significant. Results for the July sampling only are presented here. Independent data for the chlorophyll content of beet are not available for comparison, but plants given nitrate at normal levels of molybdenum and basal levels of heavy metals were normal in appearance and vigorous. The values found were therefore probably normal. They ranged from 132 to 187 mg/100 g. in young leaves and 75–137 mg./100 g. fresh weight in old leaves for sugar beet, and 106–117 mg./100 g. fresh weight for spinach beet for whole 'tops'.

The results in sugar beet of replacing nitrate by urea at the Mo_1 level were complex. Plants were initially darker green, but those given Cr^{+++}, CrO_4^{--}, Cu^{++}, or Zn^{++} had less chlorophyll in young leaves and more in the old leaves than plants in the nitrate series. The chlorotic pattern was also modified in some instances. Veins appeared darker green and the general effect was a diffuse 'speckling' rather than the clearly defined intervenal chlorosis shown by the nitrate plants. In the presence of CrO_4^{--} urea resulted in acute chlorosis of young leaves, greatly in excess of that seen with nitrate.

The effects of extra molybdenum were not appreciably changed in the presence of urea, but the chlorosis of the younger leaves was further accentuated, whilst old leaves remained relatively dark green. The chlorophyll data in Table I *a* and *b* suggest that the effects of molybdenum and urea were really additive and independent since the differences in the presence of the heavy metal additions were approximately equal and additive. In spinach beet the effect of ammonium sulphate resembled qualitatively that of urea in increasing chlorophyll content, though not significantly, in the whole 'tops' of plants *not* given excess metals. Chlorophyll was decreased significantly to a still greater extent by ammonium sulphate in the presence of manganese and zinc, but this effect was less noticeable (Zn^{++}) or nil (Mn^{++}) in the presence of high molybdenum in contrast with the additive and independent effects found for urea given to sugar beet.

Magnesium fractions

The identification of magnesium protoporphyrin and protoporphyrin 9 as probable chlorophyll precursors in *Chlorella* mutants by Granick (1948, 1948*a*) and the relation of ether-soluble magnesium fractions to chlorophyll synthesis studied by Smith (1947) suggested an examination of some magnesium fractions in this work. The results of analyses for spinach beet are given as major effects with their probabilities in Table II.

TABLE II

Significance of heavy metal, iron molybdenum, and nitrogen treatment effects on some magnesium fractions in spinach beet

Comparison of paired treatment effects on specified magnesium fractions	Determining factors	Probability
Chlorophyll magnesium		
Less with excess Mn than basal levels	Ferric iron and high Mo or NH_4	0·01
Less ,, ,, Zn ,, ,, ,,	High Mo or NH_4	0·05
Less ,, ,, Cu ,, ,, ,,	NO_3 (no data for NH_4)	0·01
Greater with NO_3 than NH_4	Excess Mn or Zn	0·01
Greater with ferrous iron than ferric	Excess Mn with NH_4 or high Mo	0·01
Less ,, ,, ,, ,, ,,	Basal levels with NH_4 or high Mo	0·05
Less with high Mo than normal Mo	Excess Cu	0·001
Less ,, ,, ,, ,, ,, ,,	Excess Zn with NH_4, NO_3, Ferrous and ferric iron	0·01–0·05
Less ,, ,, ,, ,, ,, ,,	Excess Mn with ferric iron	0·01
Total acetone-soluble magnesium		
Less with excess Cu than basal levels	NO_3	0·05
Greater with NO_3 than NH_4	Excess Mn or Zn	0·05
Greater with ferrous iron than ferric	Excess Mn with NH_4 or high Mo	0·05
Less with high Mo than normal Mo	Excess Mn with NO_3	0·02
Less ,, ,, ,, ,, ,, ,,	Excess Mn with ferric iron	0·01
Less ,, ,, ,, ,, ,, ,,	Excess Zn with NH_4 or ferrous iron	0·02
Acetone-soluble non-chlorophyll magnesium		
Greater with excess Mn than basic levels	NH_4 or ferrous iron	0·05–0·1
Greater ,, ,, Zn ,, ,, ,,	NH_4 or ferrous iron	0·05–0·1
Greater with NO_3 than NH_4	Excess Mn with ferric iron or normal Mo	0·01
Greater with ferrous iron than ferric	Excess Zn with NO_3	0·02
Less with high Mo than normal Mo	Excess Mn with NO_3 or ferric iron	0·05
Total magnesium		
Less with excess Zn than basal levels	—	0·01
Less ,, ,, Cu ,, ,, ,,	—	0·01
Greater with NO_3 than NH_4	Excess Zn with ferric iron or high Mo	0·05

Excess Cu^{++} reduced total, total acetone-soluble, and chlorophyll magnesium, but not the non-chlorophyll acetone-soluble fraction.

Excess Zn^{++} reduced the total magnesium and the chlorophyll fraction with high molybdenum or ammonium nitrogen. Total acetone-soluble magnesium was not affected and non-chlorophyll acetone-soluble magnesium was increased, but this just failed to attain significance at the 5 per cent. level.

Excess Mn^{++} did not affect the total and total acetone-soluble-magnesium fractions. Chlorophyll magnesium was reduced in the presence of ferric iron, extra molybdenum, or ammonium nitrogen and the non-chlorophyll acetone-soluble magnesium was consistently increased, but this effect just failed to attain significance at the 5 per cent. level.

The supply of iron in the ferrous instead of the ferric form increased both chlorophyll and total acetone-soluble magnesium in the presence of excess manganese with either high molybdenum or ammonium nitrogen, and increased chlorophyll in the basal treatment with these combinations. Non-chlorophyll acetone-soluble magnesium was also increased in the presence of excess zinc with nitrate.

Ammonium nitrogen, as compared with nitrate, consistently and significantly depressed both the chlorophyll and total acetone-soluble fractions in the presence of excess manganese or zinc. The non-chlorophyll acetone-soluble fraction was mainly reduced by ammonium nitrogen in the presence of manganese with normal molybdenum or ferric iron.

Extra molybdenum consistently depressed chlorophyll and decreased all soluble magnesium fractions in the presence of excess manganese with nitrate or ferric iron; chlorophyll and total acetone-soluble magnesium were reduced by extra molybdenum in the presence of excess zinc with nitrate or ferrous iron and in the presence of ammonium sulphate.

The interactions between N, Fe, and Mo on the concentration of the magnesium fractions were frequently significant and the more notable ones are outlined below. Extra molybdenum decreased chlorophyll and total acetone-soluble magnesium with excess manganese or zinc in the presence of ferric iron, but this was less marked in the presence of ferrous iron. More chlorophyll was produced in ammonium sulphate than in nitrate at the basal levels of the heavy metals, but in the presence of zinc and manganese there was more chlorosis in ammonium series. A similar trend occurred with the acetone-soluble magnesium fractions. Ferric iron promoted more chlorophyll and acetone-soluble magnesium compounds than ferrous iron in the presence of normal molybdenum, nitrate nitrogen, and excess manganese, but the reverse effect occurred in the presence of ammonium nitrogen. An opposite interaction between iron and nitrogen occurred with excess zinc at the high molybdenum level. The relationship between iron and molybdenum noted for excess manganese on total acetone soluble magnesium was also reversed in sign in the basal manganese treatments. It was noteworthy that extra molybdenum showed less significant effects when combined with ammonium sulphate, in contrast to the results obtained with urea in the previous experiment.

The chlorophyll and total acetone-soluble magnesium fractions comprised about 2·5–5 and 4–9 per cent. respectively of the total magnesium content, and with extra Cu^{++} or Zn^{++} the total magnesium concentration in dry matter ranged from 0·21 to 0·33 per cent. and was probably close to the threshold of deficiency, which in many plants is about 0·24 per cent., as compared to normal values around 0·4–0·56 per cent. found in the other treatments.

DISCUSSION

The consistently increased chlorosis caused by extra molybdenum was opposite to the results reported by Millikan (1947), which were, however, obtained with flax in water culture. It is not possible at present to say whether these two factors may account for such a striking contrast in results obtained in otherwise similar circumstances. Millikan (1950) did not, however, make quantitative determinations of chlorophyll content. He concluded in some instances that the 'golden yellow pigment' produced in flax was not an indication of induced iron deficiency and was not prevented by extra iron supplies. The colour was accentuated by molybdenum and manganese together and was thought to be similar to the molybdenum excess effect described by Warington (1937). Evidently loss of chlorophyll found in the present work and production of the yellow pigment reported by Millikan might be confused, but lack of data on chlorophyll in flax prevents further comparison at present. Warington (1951) also concluded, however, that iron-deficiency chlorosis in soya bean and flax was accentuated by molybdenum in the presence of manganese whereas vanadium did not show this effect.

As the part played by molybdenum in accentuating chlorosis is still entirely obscure, further discussion would be premature.

The apparent accumulation of a non-chlorophyll acetone-soluble magnesium fraction in the presence of excess manganese or zinc might indicate that these metals prevented the conversion of a precursor, possibly allied to protochlorophyll, to chlorophyll itself. The data do not, however, suggest this conclusion for copper. This metal sharply reduced the total acetone-soluble fraction and may therefore have interfered at an earlier stage by preventing the formation of the acetone-soluble magnesium compounds, either by competing with the magnesium itself or by antagonizing magnesium uptake—an effect also caused by zinc.

Ferrous iron usually favoured higher concentrations of each magnesium fraction in the presence of excess manganese or zinc and also, though less significantly, increased total magnesium content in all treatments. This response might be regarded as supporting previous suggestions, criticized by Arnon (1943) on grounds of insufficient evidence, that ferrous iron favoured chlorophyll formation.

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The Compositions and Quantitative Relations of Protein and Related Fractions in developing Root Cells

C. MORGAN
Botany Dept., The University, Leeds
AND
W. S. REITH[1]
Dept. of Organic Chemistry, The University, Leeds

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SUMMARY

In this investigation the amino-acid composition and the quantitative levels of the free amino acid, protein, and peptide fractions at different distances from the apex of the root have been examined. It has been shown that as the cell grows there is a threefold increase in protein, a tenfold increase in free amino-acids, and a twentyfold increase in peptides. Simultaneously the amino-acid composition of each fraction also probably changes. Further, it has been found that various fractions from the meristematic zone yield on chromatograms a variety of ninhydrin-reacting substances which have not been found in detectable amounts in extracts from other regions.

The significance of these results is discussed.

INTRODUCTION

The purpose of this investigation is the analysis of the relation between cell growth in an intact organ and the levels and composition of the protein, peptide, and free amino-acid fractions.

Since proteins are important constituents of the cytoskeleton and the primary components of enzymes it is evident that there must be a close relation in all living systems between the level and composition of protein and cellular activity. This relation is likely to be as significant a factor in the growing as it is in the mature cell, and an examination of it in the expanding cell is clearly of immediate relevance to the elucidation of the mechanism of plant cell growth. The investigation of this topic, however, requires to be developed with regard to two important aspects: (1) the relation is likely to be a reciprocal one, and (2) it is likely to involve qualitative as well as quantitative features.

That the state of the protein complex may determine the growth reaction requires little emphasis. The protein content, for instance, may regulate the total enzyme level and thus control the energy consumption in growth. At the same time it is conceivable that a reciprocal set of effects may be involved.

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[1] Present address: M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W.12.

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During growth the physical state of the system is changing. The ratio between protoplast area and volume is changing and different phase boundary systems are being developed. Further, it is not improbable that during growth, changes in the metabolic pattern are occurring. Clearly such physical and metabolic changes may well affect the conditions for synthesis and degradation of proteins and thus influence the protein content.

The importance of qualitative aspects is suggested by the changes in functional status that occur during the normal development of the cell. Normally the cell changes from a small non-vacuolated isodiametric system to one which has a twentyfold greater volume, is provided with a large central vacuole, and a permanent cell-wall. In the course of this development the capacity to divide is suppressed. Whereas the non-vacuolated cell may divide actively, the fully expanded cell does so only rarely and sporadically. Clearly it is improbable that as extensive a change as this is not accompanied by corresponding changes in the qualitative state of the protein complex as well as in its quantitative level.

These considerations have determined the design of this experimental series. The possible effect of the protein on growth has been studied in its quantitative and qualitative aspects by determining the level and amino-acid composition of the protein at four different stages of growth. The possible effects of growth on the protein complex have been explored by investigating the levels and compositions of the peptide and amino-acid fractions as well as of the protein at three different stages of growth. It is evident that if the conditions of growth are affecting the protein then relative changes in rates of synthesis and degradation affect the relative levels of the free amino-acids and peptides. Further, if simple changes in synthesis and degradation are involved, then the qualitative composition of the peptides and amino-acids should be similar to that of the proteins. It is recognized that the analysis of the amino-acid composition of various fractions and particularly of the proteins does not exhaust the examination of possible qualitative differences. Physical states may also be involved and they may also affect the situation. Nevertheless it is clear that the composition of the protein must be an important property of the system.

Sections have been cut at increasing distances from the apex of the root, observations have been made on the sections and the quantitative data related to the numbers of cells in the sections. In principle this general technique was developed by Brown and Broadbent (1950). The serial values thus obtained represent successive stages in the development of an average cell. The implications and limitations of the technique are discussed by Brown, Reith, and Robinson (1952). In this investigation we have used bean roots since these have larger growing zones than the pea roots used by Brown and Broadbent. Also we have not used serial sections as did the earlier workers. The prolonged chemical procedures involved in the investigation have necessitated a restriction of the observations to four sections taken from representative regions of the growing zone. The sections we have used with the number of cells each contains are as follows: I, taken 1·4 to 2·6 mm. from apex with 105,000 cells; II, taken 3·2 to 4·8 mm. from apex with 75,000 cells; III, taken 5·4 to 6·6 mm. from apex with 33,000 cells; IV, taken 7·6 to 8·8 mm. from apex with 21,000 cells.

The first section is taken from the meristematic region, the second from that of early vacuolation, the third from that of active growth, and the fourth from that in which growth in length is ceasing. The relative positions of the different sections is shown in Fig. 1 which gives the numbers of cells in successive 1·0-mm. zones.

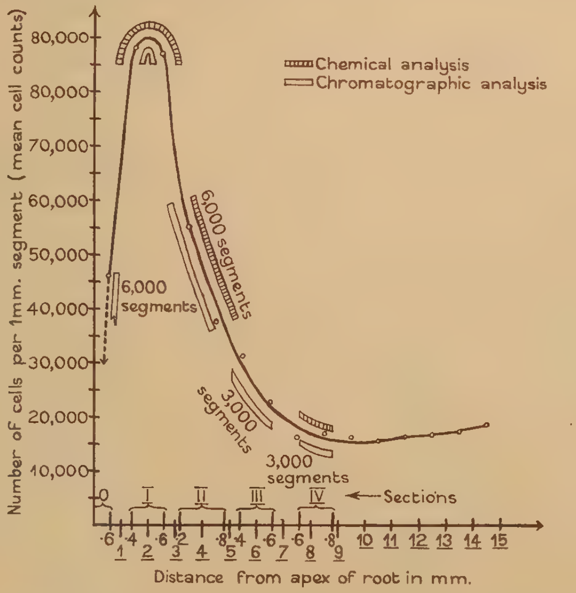

FIG. 1. Change in number of cells in successive 1·0 mm. zones in root of bean. Blocked areas refer to zones used for chemical analysis.

Quantitative estimations for protein, free amino-acids, and peptides have been made on sections I, II, and IV. The qualitative composition of each fraction has been examined chromatographically in all four sections.

It is clear that the technique we have used here only gives an indication of coincident changes in growth and in the levels and composition of the fractions studied. It does not yield data from which firm conclusions can be drawn regarding causal mechanisms. The coincident changes, however, suggest possible mechanisms, and provide the basis for further experimental work. Also they display the full scope of the process.

We wish to emphasize that some of the results presented here are only of a preliminary nature. Many of the observations are only based on single samples. The samples, however, are large and many of the results are sufficiently clear cut to warrant this report.

MATERIALS AND METHODS

Preparation of the sections. Bean roots are being used extensively in this laboratory in a variety of investigations and the standard procedure which has also been adopted by us which comprises the culture of the parent roots, cutting the sections, and determining the number of cells in each section is described by Robinson and Brown (1952).

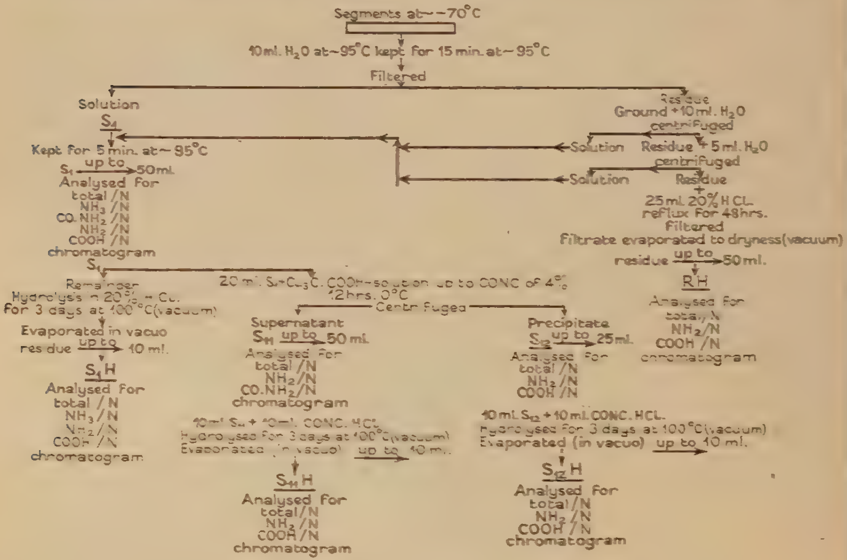

FIG. 2. Outline of fractionation procedure. For explanation see text.

The design of this investigation has required the use of an abnormally large number of corresponding sections from each region of the growing zone. For each of the first and second regions we have used 6,000 sections, and for each of the third and fourth, 3,000. The whole group has not been accumulated on a single day. On each day 400 roots have been used yielding a corresponding number of sections from each of the selected regions. After cutting, the sections are transferred to solid carbon dioxide and stored at −70° C. All the material used in this investigation has been assembled prior to starting analytical work, and in some cases the sections have been stored at −70° for some months before being used.

Fractionation procedure. The fractionation procedure is outlined diagrammatically in Fig. 2.

Each group of sections has been treated in the same way. After withdrawal from solid CO_2 they are boiled in 10 ml. of water for 15 minutes. This treatment inactivates all the enzymes which might affect the relative distribution of amino-acids in different fractions. The segments are separated from the water in which they are boiled, and ground in a mortar in 10 ml. of fresh distilled water. The suspension is centrifuged and the supernatant added to the fluid from the preliminary boiling. The residue is resuspended in distilled water, centrifuged, and the supernatant again added to the fluid fraction. As a result of this treatment two fractions are obtained, the residue R that contains all the proteins that are coagulated by boiling and a solution, S_1, which carries all the non-coagulable proteins, the peptides, and the uncombined amino-acids.

In further treatment the residue R is refluxed with 25 ml. of 20 per cent. hydrochloric acid for 48 hours. This yields a solution which is evaporated to dryness *in vacuo*, giving a residue which is dissolved in 50 ml. of distilled water. This solution, RH, contains amino-acids released by the hydrolysis of the heat-coagulable protein.

Two aliquots are taken from the solution S_1. The first is treated with 20 per cent. HCl for 3 days at 100° C. under reduced pressure, the residue from this treatment being taken up in 10 ml. of water to give solution S_1H. The second is treated in 4 per cent. trichloracetic acid for 12 hours at 0° C. A precipitate forms which is separated by centrifugation. The precipitate is suspended in water to give a fraction S_{12}. The supernatant from the centrifugation gives the fraction S_{11}. Both fractions are hydrolysed by the procedure described for S_1 to give the fractions $S_{11}H$ and $S_{12}H$. The trichloracetic acid precipitates the non-heat coagulable proteins contained in S_1, and $S_{12}H$ therefore contains only amino-acids from this material. S_{11} contains the amino-acids and peptides of S_1, and $S_{11}H$ therefore contains amino-acids released from the peptides and the amino-acids derived from the original solution.

In each of the fractions obtained by the procedure described above, the free α-amino-acid content has been determined. It is clear that from the differences between these determinations the protein, peptide, and free α-amino-acids may be estimated. The combined values from the direct hydrolisates (RH and S_1H) give the total α-amino nitrogen in all forms The value from the unhydrolysed S_1 fraction gives the total quantity of uncombined amino-acids. Thus the difference between these two sets of values is a measure of the α-amino-acid fraction in peptide bonds of all forms. The combined values from the hydrolysed R and S_{12} fractions are a measure of the total protein, and the difference between the total protein and the total peptide bond values is a measure of the quantity of peptides.

Analytical methods. Each of the fluids containing soluble components obtained in the course of the fractionation procedure has been examined for total nitrogen, α-amino nitrogen, and α-carboxy-amino nitrogen. In addition ammonia has been determined in the S_1 and S_1H fractions and amide in the S_1 solution. All soluble preparations have been examined chromatographically.

Total nitrogen has been determined by the Chibnall, Rees, and Williams (1943) modification of the micro-Kjeldahl technique, ammonia being distilled into weak acid in the apparatus of Parnas and Heller (1924) and titrated with 0·01 N. sodium hydroxide, a screened methyl red indicator being used (Tristam, 1941).

α-amino-N (NH_2 N) has been determined by the manometric Van Slyke (1929) method. The values obtained by this method are subject to a considerable error that cannot be evaluated with accuracy. The error is due to the presence of NH_3, lysine, guanidyl, and probably phenolic groups. These amino N determinations, however, may be used for comparison with the COOH N determinations.

α-carboxy-amino-N (COOH N) has been determined by the manometric ninhydrin-CO_2 method of Van Slyke, Dillon, McFadyen, and Hamilton (1941). It is probable that the only error involved in this estimation is due to aspartic acid, which yields 2 moles of CO_2 instead of the normal 1.

The chromatographic examinations have been carried out by the two-dimensional method of paper chromatography of Consden, Gordon, and Martin (1944) as modified by Dent (1948). After desalting the extracts or hydrolysates, aliquots containing about 200 μg. of COOH N are run first with phenol and then with lutidine, the development of the chromatograms with ninhydrin being effected, after steaming, with a stream of hot air.

EXPERIMENTAL RESULTS

Analytical data. The analytical data are presented in two forms. In Table I they are given in terms of g. per 6,000 sections and in Table II in terms of g. per cell in each of the sections. The free α-amino acid, peptide, and protein values of Table II are reproduced in Fig. 3.

Tables I and II give experimentally determined values and derivative values calculated according to the procedure set out in the previous section. The first horizontal series gives total nitrogen values determined by the Kjeldahl technique and the second total α-amino nitrogen determined after hydrolysis of the different fractions. It is significant that there is a large and persistent difference between these two sets of values. Such a difference might be expected, since all the nitrogen in the tissue is certainly not in the form of α-amino nitrogen, and furthermore the analytical procedure undoubtedly involves a destruction of certain amino-acids. At the same time the extent of the difference emphasizes the importance of determining protein in terms of α-amino nitrogen. It is clear that all fractions contain large quantities of non-α-amino nitrogen, and determinations based on differences in quantities of total nitrogen are likely to involve considerable errors.

In this connexion it may be significant that the difference is given in the S_{12} as well as in the R and S_1 fractions. The values of S_{12} are obtained after precipitation from S_1 with trichloracetic acid. The difference is large and

TABLE I

Composition of various fractions extracted from different groups of sections. The values are in g.× 10⁻³/6,000 sections. For explanations of symbols see text. Figures in italics have been experimentally determined, the rest are calculated values.

	Section I				Section II				Section IV			
	$R+S_1$	S_1	R	S_{12}	$R+S_1$	S_1	R	S_{12}	$R+S_1$	S_1	R	S_{12}
I. Total N	127·5	*37·0*	*90·5*	*6·3*	121·0	*55·2*	*65·8*	*5·1*	73·4	*40·6*	*32·8*	*2·4*
II. Total α-amino-N (Total COOH/N after hydrolysis)	41·7	*8·6*	*33·1*	*2·2*	63·7	*19·5*	*44·2*	*4·0*	38·6	*19·0*	*19·6*	*1·4*
III. Free α-amino-acids-N (COOH/N before hydrolysis)	5·5	*5·5*	—	*0·2*	8·6	*8·6*	—	—	12·8	*12·8*	—	—
IV. Total N in 'peptide bonds' (II–III)	36·2	3·1	33·1	2·0	55·1	10·9	44·2	4·0	25·8	6·2	19·6	1·4
V. Total protein α-amino-N (COOH/N after hydrolysis in R and S_{12})	35·1	2·0	33·1	2·0	48·2	4·0	44·2	4·0	21·0	1·4	19·6	1·4
VI. Total peptide α-amino-N (IV–V)	1·1	1·1	—	—	6·9	6·9	—	—	4·8	4·8	—	—

TABLE II

Values of Table I calculated to unit cell basis (g.× 10⁻¹⁰)

	Section I				Section II				Section IV			
	$R+S_1$	S_1	R	S_{12}	$R+S_1$	S_1	R	S_{12}	$R+S_1$	S_1	R	S_{12}
I. Total N	2·02	0·59	1·44	0·10	2·69	1·23	1·46	0·11	5·82	3·22	2·6	0·19
II. Total α-amino-N	0·66	0·14	0·53	0·04	1·42	0·43	0·98	0·09	3·06	1·51	1·55	0·11
III. Free α-amino-acids	0·09	0·09	—	0·003	0·19	0·19	—	—	1·02	1·02	—	—
IV. Total α-amino-N in 'peptide bonds'	0·57	0·05	0·53	0·03	1·22	0·24	0·98	0·09	2·05	0·49	1·56	0·11
V. Total protein α-amino-N	0·56	0·03	0·53	0·03	1·07	0·09	0·98	0·09	1·67	0·11	1·56	0·11
VI. Total peptide α-amino-N	0·016	0·016	—	—	0·15	0·15	—	—	0·38	0·38	—	—

suggests that trichloroacetic acid precipitation may in certain instances give exaggerated values for total protein.

If the relative composition of the tissue remains constant, with a standard procedure the difference between total and α-amino nitrogen values might be expected to remain constant. It is significant, however, that in the present array of data the relative difference is greatest in the first section and tends to be less in the second and fourth section. This suggests that there may be a greater relative accumulation of certain non-α-amino nitrogen compounds in the first than in the other two sections.

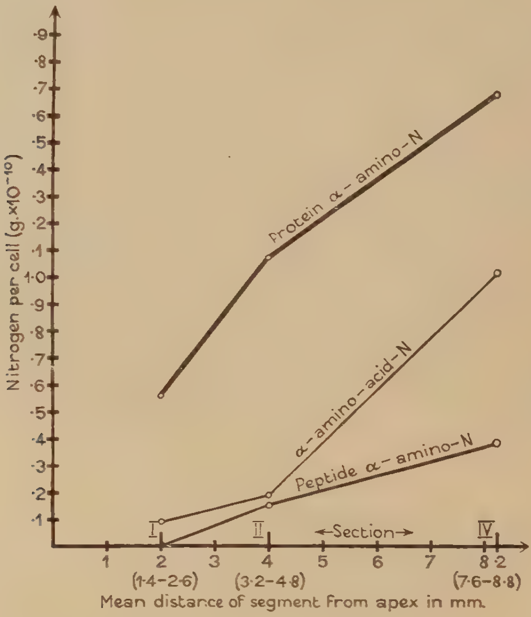

FIG. 3. Change per cell in various α-amino-acid containing fractions.

In relation to the development of the cell the data of Table II and Fig. 3 are the most relevant. It may be noted that all fractions increase from the first to the second section, and from the second to the fourth. Also the relative increases are not the same. From the first to the fourth section there is a threefold increase in protein, a tenfold increase in free amino-acids, and about a twentyfold increase in peptides.

The present series of results with respect to the protein content of the cell are broadly in agreement with those of earlier workers who used the total nitrogen of various precipitates. Blank and Frey-Wyssling (1941), Kopp (1944), and Brown and Broadbent (1950) all reported an increase in protein during growth in intact organs.

Chromatographic data. All the solutions obtained in the normal fractionation procedure were examined chromatographically. On various chromatograms many of the spots could be identified as being due to known amino-acids, many of them, however, could not. The data regarding spots of known origin are summarized in Table III and those relating to spots of unknown character are given in Table IV. The values in both tables are scores of colour intensities and are, of course, subject to all the errors and limitations normally associated with such subjective estimates.

In Tables III and IV we give certain data (section O) which are obtained from the first 0·6 mm. of the root. This section is largely occupied by root-cap tissue.

The data of Table III show that certain amino-acids are not present in detectable amounts in all preparations. The most notable of these are glutamine, arginine, and tryptophane. Glutamine may have been present in the free state, and the amide group may have been destroyed either during the preliminary boiling or during desalting. Similarly with arginine it is probably hydrolysed during desalting, and the significance of ornithine is probably that it represents a product of arginine cleavage. A spot for tryptophane might have been expected, however, if this acid had been present in detectable amounts in the aqueous extracts. Asparagine is present only in the unhydrolysed preparations, but this is no doubt due to the degradation of the amide group during acid hydrolysis. Finally, it is evident that there is a large accumulation of dicarboxylic acids in all preparations of sections I, II, III, and IV, although the absence of aspartic acid from section O may be noted.

There are certain important differences between the sections. Cysteic acid is present at least in some preparations of sections I and II, but tends to be absent from those of III and IV, and ornithine is present in all preparations of section I but tends to be absent from those of all other sections of the main series.

The spots of unknown origin are listed in Table IV in an alphabetical series. They are given in this form since although those that recurred did so in the same relative positions, they were frequently displaced by other substances, and the *Rf* values could not in all cases be determined. All unidentified spots that occurred in the series of chromatograms have been listed. It is doubtful, however, if any significance can be attached to those that have only been found once or twice in the whole series of observations. The possibilities have been examined that certain spots are due to unhydrolysed peptides or that they are due to products of prolonged storage. Both possibilities may be disregarded since rehydrolysis of the RH residue by heating *in vacuo* with 20 per cent. HCl at 100° C. does not alter the situation significantly and chromatograms prepared from freshly excised material give results similar to those obtained with stored material. The results of this group of observations are given in Table V.

The data of Table IV show that while the spots C, L_1, L_2, P, Q, T, X, Y, and Z occur only in hydrolysates, all others occur both in hydrolysed and

TABLE III

Results of chromatographic analysis of various extracts from different sections. For explanation of symbols see text

Ft. = Faint; Scr. = Screened

Amino acid	Section O		Section I						Section II						Section III					Section IV				
	S_1H	RH	S_1	S_1H	S_{11}	* $S_{11}H$	$S_{12}H$	* RH	S_1	S_1H	S_{11}	$S_{11}H$	$S_{12}H$	RH	S_1	S_{11}	$S_{11}H$	$S_{12}H$	RH	S_1	S_1H	* $S_{11}H$	$S_{12}H$	* RH
1. Aspartic acid		—	2	3½	3	5	3½	3	3	3	2	1½	3	2½	1	5	5	½	1	—	3	5	2½	1
2. Glutamic acid	4½	2½	4	4	5	6	4	4	3	5	3	4	4	1½	6	5	5	1	2	½	2	5	1	½
3. Glycine	4	3	Scr.	3½	Scr.	4	3½	3½	Scr.	4	Scr.	3½	3	2½	Scr.	Scr.	2	2	3	Scr.	3	3	3½	1½
4. Serine	4	2	2½	1½	3	4	3½	3	2½	3	1½	2½	3	2	3	1½	2	1	3	2½	2½	3	3½	1
5. Asparagine		—	2½		2	—	—	—	2½	—	2½	—	—	—	4	3	—	—	—	3				—
6. Alanine	4	3	5	4	4	5	3½	2½	4	5	3½	4	3½	3	6	4	3	2	4	3½	3	2½	3	2
7. Proline	2	—	1	1½	½	1½	1	1	1	1	1½	1½	2½	1½	—	—	1	Ft.	½	—	2½	2½	2½	1
8. Ornithine	Ft.	Ft.	1½	1	1	1	½–1	1	—	—	—	—	—	½	—	—	—	—	—	—	—	Ft.		
9. Lysine	3½	3	2	2½	2	2	3	3½	1	2	1	2	3	3	—	—	1½	1	4	1	2½	1½	2	½
10. α-amino-butyric acid	½	½	1	2	Ft.	1	1	1	—	½	1	½	2	1	—	—	—	—	½	Ft.	1	2	½	—
11. Valine	3½	3	2½	3	2½	3	3½	3	2	4	1½	2	3½	3	2	1½	2½	3	4	2½	3½	4	3	1½
12. Leucines	4	4	2½	3	2	4	3½	5	2	4	2	3	3½	3½	1	½	2	3	5	2	4	4	3½	3
13. Phenyl alanine	1	½	½	1	1½	1	½	2½	1	1½	1	½	2½	1½	1	½	1	½	1	1	2	2	1½	1½
14. Tyrosine	2	1	3	3	2	3	2	1	1½	1½	2	2½	2	1	6	2	2	½	1	2	2½	2½	1½	1½
15. Threonine	Ft.	½	Ft.	1½	—	1	2½	½	1	1½	1½	½	2½	1½	½	½	1	Ft.	2	—	2	½	1½	—
16. Cysteic acid	1	1	1	½	½	1½	½	½		–	2	1½	½	½	Ft.	—	—	—	3	–				
17. Arginine	?	?	1								Ft.	½	—		—	—	—	—	½					
18. Hydroxy-lysine																								Ft.
19. Djencolic acid																								
20. 'Slow' serine		½	—											Ft.	—	—								

TABLE IV

Distribution in different extracts of unknown ninhydrin reacting substances. For explanation of symbols see text

Ft. = Faint; Tr. = Trace

Novel spot	Section O		Section I						Section II						Section III					Section IV				
						*		*														*	*	*
	S_1H	RH	S_1	S_1H	S_{11}	$S_{11}H$	$S_{12}H$	RH	S_1	S_1H	S_{11}	$S_{11}H$	$S_{12}H$	RH	S_1	S_{11}	$S_{11}H$	$S_{12}H$	RH	S_1	S_1H	$S_{11}H$	$S_{12}H$	RH
A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Ft.	—	—	—	—	—	—	—	—	—
A or C	—	—	—	—	—	—	—	—	—	1	—	1	1	—	—	—	—	—	—	—	—	—	—	—
B	1½	—	2½	2	Ft.	2	½	½	1	2	1½	2	1½.	1	—	—	Ft.	—	1½	1	—	1½	½	½
C	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	Ft.	—		—	—	—
D	Ft.	—	1	1½	1½	½	—	½	Ft.	1	½	1½	½				—	—	—	½	½	1½	—	—
E	—	—	1	½	1½	1½	—	—	½	1	1	½	—	1½	—	—	—	—	—	—	—	—	—	—
F	—	—	—	—	—	—	—	—	1	Ft.	Ft.	Ft.	Ft.	—	—	—	—	—	—	—	—	—	—	½
G	1	½	½	½	2	1	1	½	1	1	1	—	1	1	—	—	—	—	—	—	—	Ft.	Ft.	1½
H	1	½	½	½	1½	1½	½	2	1	—	1	1	—	½	—	—	—	—		—	—		—	—
I	—	—	—	—	—	—	—	½	—	Ft.	—	—	—	—	—	—	—	—	—	—		—	—	
J	—	—	½	½	—	—	—	½	—	—	½	½	½	1	—	—	—	—	—	—	—	—	—	—
K	½	1	1	1	—	—	½	½	—	—	½	—	½	1½	—	—	—	—	—	—	½	3	—	—
L	1	1	—	—	2	1½	—	—	—	—	1	Ft.	1½	1½	—	—	—	—	—	—	—	—	—	1½
L_1	—	—	—	—	—	—	—	½	—	—		—	—	—		—	—	—	—			—	—	—
L_2	1½	1	—	—	—	—	—	½	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—
N	Tr.	½	½	1	1½	1½	—	1	—	—	1	1½	1	1½	—	—	—	—	—	Ft.	—	—	—	
O	—	—	½	1	—	—	—	—	—	—	1	1	1	1		—	—	—				—		—
P	—	—	—	—	—	—	—	—	—	—	—	Ft.	Ft.	1½	—	—	—	Ft.	—	—	—	—	—	1
Q	—	—	—	—	—	—	—	—	—	—	—	—	½		—	—	—		—	—	—	—	—	—
R	—	—	1½	—	—	—	—	—	—	—	—	—	—	Ft.	—	—	—	—	—	—	1½	Ft.	—	1
S	—	—	1	1	1	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	1	2
T	—	—	—	—	—	½	—	—	—	—	—	—	—	1½	—	—	—	—	—	—	—		—	—
U	Tr.	—	½	½	1½	1½	½	½	—	—	—	—	—	1½	—			—	—			—	—	
V	—	—	1	1½	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	—	—	—	—
W	—	½	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
X	—	—	—	—	—	1½	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Y	—	—	—	—	—	1	1½	½	—	—	—	—	—	—	—	—	—	—		—	—	—	—	—
Z	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

unhydrolysed solutions. It is an important feature of the situation that the spots of unknown origin are more abundant in chromatograms from sections I and II than they are in those from sections III and IV. The general tendency is for these unknown substances to be relatively more abundant in young tissue and for them to disappear as the tissue matures.

We wish to emphasize that although the spots must be due to substances that react with ninhydrin, we have no evidence other than this that the substances are amino-acids. Further, although at least some of them may be amino-acids and may be incorporated into the protein, again we have no evidence that such is the case. The release of some of the substances on hydrolysis may be due to the breaking down of the adsorbent to which they have been attached.

TABLE V

Results of chromatographic examination of extracts from freshly cut sections and of rehydrolysates

SECTION I. FRACTION *R*

Amino-acids	Hydrolysate *RH*	Freshly cut sections	Rehydrolysate	Novel spots	Hydrolysate *RH*	Freshly cut sections	Rehydrolysate
1. Aspartic acid	3	3	2	B	½	1	1
2. Glutamic acid	4	3	2	C	—	½	1
3. Glycine	3½	3	4	D	½	1	1
4. Serine	3	2½	3½	E	—	—	Faint
5. Asparagine	—	—	—	G	½	½	1
6. Alanine	2½	2½	4½	H	2	½	½
7. Proline	1	½	1	I	—	—	Faint
8. Ornithine	1	Faint	½	J	½	1	Faint
9. Lysine	3½	3½	4	K	½	½	½
10. α-Amino-butyric acid	1	½	—	L	—	—	2
11. Valine	3	3	4½	L_1	½	½	—
12. Leucines	5	4	5	L_2	½	½	Faint
13. Phenyl alanine	2½	½	1½	N	1	½	—
14. Tyrosine	1	½	2½	O	—	½	½
15. Threonine	½	1	3	S	—	—	½
16. Cysteic acid	½	½	1	T	—	—	1½
17. Arginine	—	—	1	U	½	—	—
18. Hydroxy-lysine	—	—	—	V	—	½	1
19. Djencolic acid	1	Faint	—	Y	—	1	—

Diagrams from sample chromatograms (those marked with an asterisk in Tables III and IV) are given in Fig. 4.

DISCUSSION

The interpretation of both the analytical and the chromatographic data depends on the significance that can be attached to the over-all qualitative changes in the protein complex. Two possibilities must be considered. The changes may represent a developing situation in the cytoplasm in the sense that as the cytoplasmic protein in the cell increases its composition changes. Or the changes may simply represent corresponding modifications in the nuclear:cytoplasmic ratio. The protein content of the nucleus relative to that of the cytoplasm is unknown and it is uncertain as to whether the nucleus grows with the cytoplasm. It may be that the amino-acid composition of nuclear protein is different from that of cytoplasmic protein, that both do not change, and that the ratio of cytoplasmic to nuclear protein increases as growth proceeds. The result of such a situation with the procedure used here might be an apparent absolute decrease in the percentage contribution of certain components to the over-all protein complex of the cell. While this possibility cannot be excluded on the basis of any data at present available, certain observations suggest that the over-all qualitative changes are in fact an expression of changing conditions in the cytoplasm. While many of the differences in amino-acid composition have been found in both hydrolysed and unhydrolysed extracts, preliminary observations suggest that a free aqueous phase is not present in the nuclei of bean roots. Secondly, that while the increase in protein per cell between the first and second sections is not great, nevertheless there is a considerable difference in pattern in certain respects between the two sections.

Thus it may be accepted tentatively that as the cell enlarges up to the stage at which growth in length ceases, protein, free amino-acids and peptides increase, while at the same time certain important qualitative changes occur in all the amino-acid-containing fractions of the cytoplasm. In the meristematic stage these yield cysteic acid, arginine, and possibly a variety of unknown ninhydrin-reacting substances. As the cell enlarges and matures these substances tend to decrease relatively to other cytoplasmic components.

The changing quantitative and qualitative relations carry two sets of implications. They may be considered in terms of effects on growth, and in terms of the reciprocal relation of effects of development on the complex of nitrogenous substances.

It is possible, although improbable, that the increasing protein content as such controls the growth of the cell. On the other hand, it is highly probable that the increase in protein occasions a corresponding change in the level of the enzyme complex of the cell, and thus provides for a more intensive metabolic exchange, which in turn may be required in the continuance of growth. While protein increases, however, its amino-acid composition changes. This might be expected to promote a corresponding change in the enzyme pattern. In this connexion it may be of some significance that the changes in the composition that accompany growth are also associated with changes in functional status. When the cell is fully expanded and does not normally divide, the protein it contains carries relatively less cysteic acid, arginine, and possibly unknown ninhydrin-reacting compounds than does the protein of non-vacuolated meristematic cells. Clearly it may be that division depends on a particular protein complex. It may be noted that Hammett (1929) has already provided evidence that *SH* compounds are involved in division in the bean root as well as in other tissues.

Certain of the data suggest that the growth process itself may influence

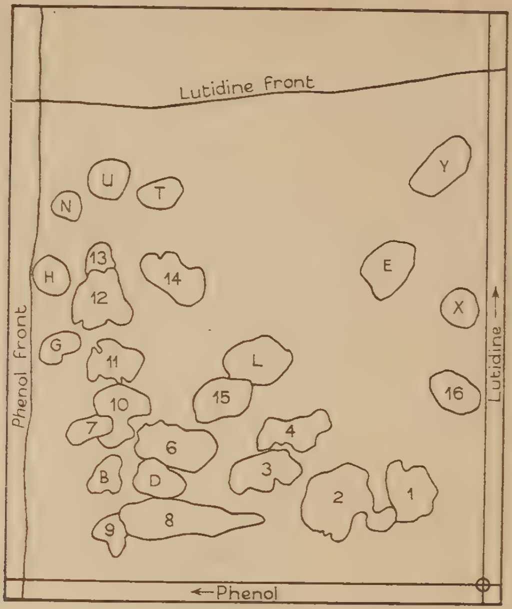
Lutidine front
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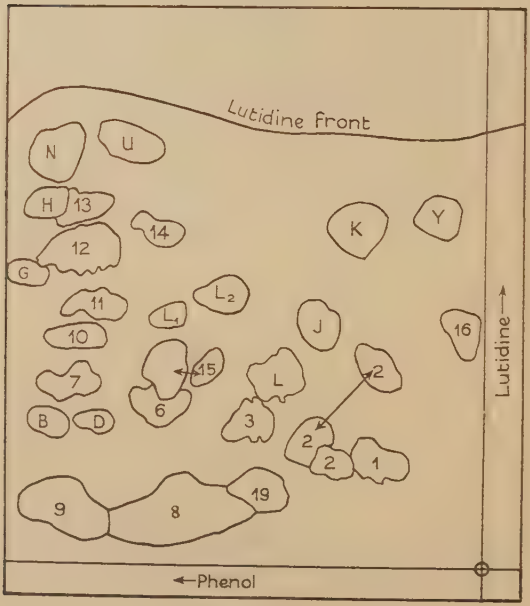
FIG. 4 (*a*).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K
Y
12
G
L₂
11
L₁
J
16
10
Lutidine →
15
2
7
L
6
3
B
D
2
2
1
19
9
8
← Phenol

FIG. 4 (*b*).

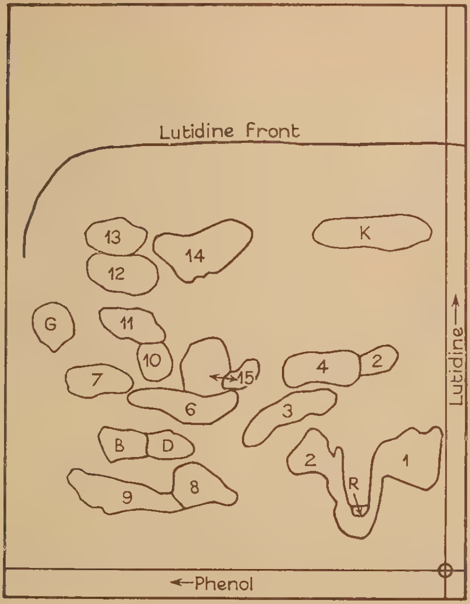

FIG. 4 (*c*).

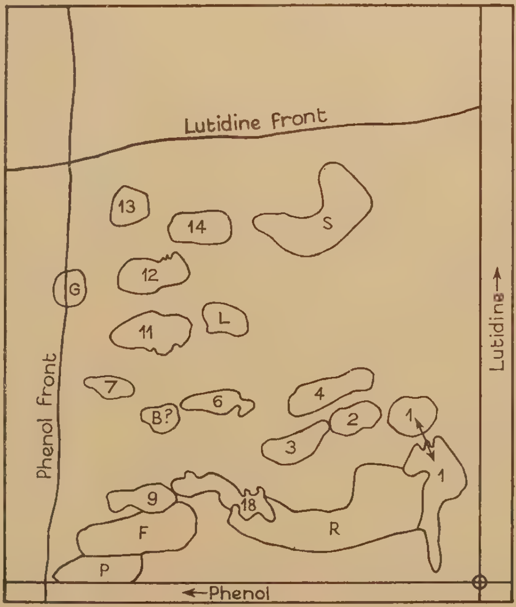

FIG. 4 (*d*).

FIG. 4. Chromatograms of different preparations from two regions of the root, (*a*) $S_{11}H$ from section I, (*b*) *RH* from section I, (*c*) $S_{11}H$ from section IV, and (*d*) *RH* from section IV.

the relative distribution and composition of the various nitrogen fractions. It is probable that the content of free α-amino nitrogen is determined simply by the conditions which control the accumulation of electrolytes in the cell. This is suggested by the fact that although the absolute level of the free amino-acid increases, nevertheless the concentration of this fraction probably remains more or less constant. Concentration values have been calculated from the present series of results and from the water content data of Robinson and Brown (1952), and in percentage terms the values for each section are:

Section I	0·55
Section II	0·45
Section IV	0·63

Clearly it is probable that as the cell enlarges the volume of the vacuole increases and provides an increasing quantity of water into which the amino-acids can be absorbed.

At the same time the data suggest that changing metabolic as well as physical states may influence the levels of the various nitrogen fractions. In a stable metabolic system in which the concentration of free amino-acids remains constant the relation of peptide to protein might also be expected to remain constant. In fact it does not. Whereas during growth there is a three-fold increase in protein, there is a twenty-fold increase in peptides. Such a change cannot be attributed to changing physical states in the system, and is more probably a reflection of a changing relation between synthesis and degradation of protein.

Further, the change in the amino-acid pattern while it probably promotes a corresponding modification in the enzyme complex is also probably an expression of a developing metabolic state. Clearly the fact that certain amino-acids and certain ninhydrin-reacting substances decrease relatively or absolutely as the growth of the cell proceeds suggests strongly that the mechanism determining the synthesis of amino-acids changes with growth. It is a matter of some interest in this connexion that the unknown ninhydrin-reacting substances in the meristem tend to decrease when the tissue is excised and cultured in a solution containing sugar and inorganic ions.

ACKNOWLEDGEMENTS

We have to thank the Agricultural Research Council for financial support of this work. We also have to thank Professor F. Challenger for allowing the chemical and chromatographic work involved in this investigation to be conducted in the Department of Organic Chemistry of Leeds University, and particularly for allowing one of us (C. M.) to work in this Department during the period of the investigation.

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The Function of Urease in *Citrullus* Seeds[1]

W. T. WILLIAMS
University of Southampton
AND
P. CH. SHARMA
Bedford College, London[2]

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SUMMARY

Urease is present in considerable quantity in the cotyledons of *Citrullus*, though elsewhere in the plant it is present only in traces or is absent; urease activity in the cotyledons changes during growth, showing an initial rise followed by an abrupt drop almost to zero. These changes, under a wide variety of conditions, are not correlated with those in the major nitrogen fractions; they are, however, closely correlated with cell extension and the associated changes in water content and respiration. A connexion with chlorophyll formation is possible but unlikely. It is suggested that the changes in cotyledonary urease constitute merely one aspect of the 'protoplasmic differentiation' that takes place as a cell matures.

INTRODUCTION

THE game of 'finding the function' has a long and honourable botanical history. Unfortunately it was pursued so zealously towards the end of the last century—sometimes to conclusions both naïve and improbable—that the teleological approach was discredited for a generation. Nevertheless, its value lies in the fact that a new or unexpected structure excites in a teleologist curiosity; in an anti-teleologist. only contemplation. It is in this belief that we reopen the problem of the occurrence of urease in plants: does this enzyme, reputedly so specific in activity, fulfil any useful function in the metabolism of the plants in which it is found? A low urease activity is widespread in plants and animals and excites little comment; but in a few species—notably in the seeds of *Canavalia* and *Soja* (Leguminosae) and of *Citrullus* (Cucurbitaceae)—the enzyme occurs in quantities so great that it can hardly be conceived as serving the sole purpose of hydrolysing urea. Indeed, one of us (Williams, 1950) has estimated that the cotyledons of *Citrullus* contain sufficient urease to decompose more than their own weight of urea every hour. The argument from quantity is of course not decisive: the tendency in nature towards unduly lavish provision of facilities was remarked by Samuel Butler in his 'Evolution Old and New' (1879). It does, however, justify us in inquiring whether the tacit assumption that urease exercises in the plant its familiar *in vitro* properties is justified: and this, broadly, is the subject of the present research.

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[1] Part of a thesis approved for the degree of Ph.D. in the University of London.
[2] Now at the University of Gauhati, Assam, India.

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Properties. An excellent account is given in Sumner and Myrbäck (1951), vol. i, pt. 2. Urease appears to be among the most specific enzymes known; apart from the recent claim by Shaw and Kistiakowsky (1950) that biuret is hydrolysed by crystalline urease, its sole activity appears to be the conversion of urea to ammonium carbonate by the addition of two molecules of water. It is now generally agreed that the hydrolysis proceeds via carbamate, and not via cyanate; it is, however, possible that spontaneous (uncatalysed) hydrolysis in aqueous solution may produce cyanate (Wyatt and Kornberg, 1952), and this possibility has been used as a basis for a theory of the function of urease in plants (*vide* below).

Occurrence. The richest of all known sources is the seed of *Canavalia ensiformis* DC.; the seeds of *Citrullus vulgaris* Schrad. contain, weight for weight, about one-eighth of this quantity (Damodaran and Sivaramakrishnan, 1937). This represents only a very small fraction of the total protein present. Sumner and Somers (1947) have calculated that the extractable urease in jack-bean (*Canavalia ensiformis*) meal does not exceed 0·15 per cent. of the total weight of protein; and the urease content of *Citrullus* seeds is presumably correspondingly smaller.

Granick, working with *Canavalia ensiformis* (1937*b*) and *Soja max* (1938), found that although urease was detectable in all parts of the plant, the great bulk of the activity resided in the cotyledons, and that this activity decreased as germination proceeded. In both *Canavalia* and *Soja* the activity tended to increase in actively dividing cells, the rate of increase falling off as extension commenced; urease-activity reached its maximum at about the end of the extension phase and then fell gradually. One of us (Williams, 1950) has already briefly reported a similar, though more abrupt, fall in the cotyledonary urease of *Citrullus* during germination.

Function. Sumner and Somers (1947) state that 'what the plant employs urease for it is difficult to say', and suggest that it may be needed to destroy urea which might otherwise accumulate in the plant. Urea is not particularly toxic but might be converted to the toxic cyanate by uncatalysed hydrolysis. (The extent to which urea and ureides occur in plants, and the mechanisms by which they might be formed, have been extensively investigated by Klein and Taubock (1931).) The theory is perhaps irrefutable, though hardly plausible: the epicotyl of *Citrullus*, for example, appears (*vide* below) to contain no urease, but does not suffer thereby when the cotyledons fall.

A second theory (cf. Klein, 1931) is that urease makes available to the plant (as ammonia) any urea which might be formed in, or absorbed by, the plant. However, if this is its main function, it is difficult to explain the great concentration within the cotyledons, which are lost early in the plant's life.

Historically the next theory is that advanced by Granick (1938); but we may conveniently leave this temporarily to examine a theory put forward by one of us (Williams, 1950) and, incidentally, advanced in ignorance of Granick's work. It was suggested that the amidase properties of urease are irrelevant to its normal function; that it is in fact part of the 'reserve' protein material of the seed, to be drawn upon as a nitrogen supply during the period before external nitrogen can be absorbed. The researches we later describe show this theory to be untenable, as already briefly reported (Williams and Sharma, 1951).

Granick's (1938) semi-quantitative studies showed that urease content is closely correlated with the stage of development of the cells in which it occurs; and he concluded that it is a 'growth-bound enzyme'. He does not explain this term; but we take it to imply that the presence of urease is connected with the normal processes of cell-growth rather than with any specific aspect of nitrogen metabolism. We may anticipate our conclusions by expressing our complete agreement with this view, though, with a considerably greater volume of quantitative data at our disposal, we believe we are able to express it less ambiguously. The evidence on which we base this agreement is set out in the pages which follow.

For a discussion on the possible role of urease in the gastric mucosa of animals, *vide* Davies and Kornberg (1951), in which earlier work of this nature is reviewed.

EXPERIMENTAL MATERIAL AND METHODS

Citrullus vulgaris Schrad. (watermelon). Seeds of eight varieties were generously provided by the Ferry-Morse Company of California; all experiments apart from those specifically concerned with varietal differences were carried out on var. 'Tom Watson'.

Urease-estimation: biochemical methods. A crude estimate was obtained where necessary by mixing neutral plant extract with a neutral aqueous solution of urea in the presence of a suitable indicator such as phenol red, then noting the depth and rapidity of development of the resulting alkali colour. We adopted the method of 'aeration-titration' rather than 'colorimetric timing' (van Slyke and Archibald, 1944).

The reacting mixture was a 3 per cent. solution of urea in 0·167 M potassium phosphate buffer at pH 7·0 containing approximately 1 per cent. of egg albumin as a protective colloid; it had been shown by Harman and Niemann (1949) that the competitive inhibition by phosphate of urease-catalysed hydrolysis of urea is negligible at such high urea concentrations. The urease extract was prepared by grinding the plant material (usually cotyledons) in glass-distilled water in a glass mortar and making up to a definite volume. Two millilitres of the extract were added to 10 ml. of the urea buffer, 2 drops of capryl alcohol added to prevent frothing, and the reaction allowed to proceed at 30° C. for 15 minutes. The reaction was then stopped by the addition of 10 ml. saturated aqueous K_2CO_3 and the ammonia determined by aeration into 25 ml. saturated boric acid, which was then titrated with 0·01 N.HCl using Ma and Zuazaga's indicator (a mixture of methyl red and brom-cresol green in 95 per cent. alcohol). A duplicate determination was carried out simultaneously, using a buffer from which the urea had been omitted.

Expression of results. It must be remembered that, although it may at times be convenient to speak of 'urease content', all measurements are strictly of urease *activity*. The units of activity as variously defined by Sumner and Hand (1928) and by Damodaran and Sivaramakrishnan (1937) were not convenient for our material; we have therefore expressed our results direct in mg. ammonia-N produced by a stated quantity of plant tissue during 15 minutes under the conditions specified above. Enough is known of the properties of urease to relate the three sets of units should it ever be desirable.

Urease: histochemical methods. Two methods are on record, the earlier due to Sen (1930); we must admit bluntly that, despite many attempts, we have completely failed to make this method work. On the other hand, the 'lake' method of Granick (1937*a*) has proved simple and effective, and is implied wherever reference is made in this paper to histochemical observations.

Nitrogen fractions. Total N and soluble N were estimated by the normal micro-Kjeldahl technique, it being assumed that the material insoluble in trichloracetic acid represented protein-N. Preliminary experiment showed that 4 hours' digestion with Chibnall's catalyst was ample. Distillation was carried out in a Markham apparatus and the ammonia collected in saturated boric acid containing Ma and Zuazaga's indicator.

Invertase was estimated by the method of Sumner and Howell (1935), the subsequent sugar estimation being carried out by Cole's ferricyanide method.

Chlorophyll was extracted with 80 per cent. acetone, transferred to ether and saponified by a solution of KOH in methyl alcohol. Water was added, and the saponified chlorophyll separated from the ethereal solution of the yellow pigments. After dilution to standard volume the chlorophyll was estimated colorimetrically by comparison with the inorganic colour standard of Guthrie (1928) in a Spekker photometer.

Dry weights were taken after 24 hours at 80° C.

Respiration rates were measured with a Warburg constant-volume respirometer.

VARIATION BETWEEN SEEDS

Apart from the observation by Annett (1914) that the urease activities of five varieties of soybean were virtually identical, nothing appears to be known about the extent of variation between seeds of the same variety or between seeds of different varieties. Some knowledge of this variation was essential for our purposes, since without it no decision could be taken as to the extent of replication or the size of seed-sample desirable. We therefore carried out a fairly extensive investigation of this nature; full details are presented in the thesis (Sharma, 1952) and only the results quoted here.

(*a*) *Between seeds of the same variety.* Ten seeds of var. 'Tom Watson' gave a mean value for urease activity, expressed as mg. ammonia-N per seed, of 4·63 with a standard error of ±0·25 and coefficient of variation 17·03 per cent. Expressed as activity per g. fresh weight of seed (excluding testa) the corresponding figures were 137·25±14·42 mg., with coefficient of variation 33·23 per cent. The 'per seed' measurement is clearly the less variable, and it appears that an approximately constant amount of urease is produced per seed, irrespective of minor variation in seed weight. The variation is not extreme (range 3·50 to 5·80 mg. NH_3—N per seed), which has enabled us to work with small samples, usually of 4 or 5 seeds.

(*b*) *Between seeds of different varieties.* Out of the 25 or so standard varieties of watermelon grown in the U.S.A., seeds of the following 8 were available to us: 'Dixie Queen', 'Florida Giant', 'Klondyke Blue Ribbon W.R.', 'Klondyke Black Seeded', 'Klondyke Brown Seeded', 'Klondyke R.7 W.R.', 'Tom Watson', and 'Wonder'. The urease activity of two replicate samples of each variety, each of 5 seeds, was measured; the whole determination was repeated on a subsequent occasion. The different varieties differ considerably in seed size, and it is presumably because of this that, as between varieties, activity per g. fresh weight was less variable than activity expressed on a 'per seed' basis. Analysis of variance showed that seven of the varieties showed no significant difference in activity per g. fresh weight: the eighth—'Dixie Queen'—was significantly low. The variety 'Tom Watson' was the second highest on a 'per seed' basis (the highest being var. 'Wonder'), and, since we already had some experience of its germination behaviour, we selected it for use in all further experiments.

A disquieting feature of the analysis was that, although variation between replicates (as measured by the 'error' term) was low, that between the two occasions was considerable and significant. Complete elucidation of the phenomenon promised to be unduly time-consuming and was not attempted; we have some reason to believe that it is partly attributable to the buffer solutions used, and may be a reflection of our difficulty in obtaining water of sufficient purity. The possibility of a change in urease-content of seeds during storage cannot, however, be ruled out.

(*c*) *Other considerations.* In three seeds of var. 'Tom Watson' the cotyledons were separated and analysed individually. The variation between cotyledons of the same seed was considerable, and in any experiment in which these are handled separately, this fact must be borne in mind as a possible additional source of variation.

We had considered it possible that there might be some relationship between urease-activity and total N in individual seeds; but preliminary investigation of a small number of seeds failed to show any such relationship and the work was not continued.

DEVELOPMENT

Time-course. Germination was very erratic if the seeds were sown direct in soil, the seedlings generally emerging between 10 and 15 days after sowing. Seeds were therefore first soaked in water for 24 hours, then transferred to a folded wet filter-paper and kept in an incubator at 25° C. With this treatment, about 70 per cent. of the seeds germinated within 24 hours, and the remainder within 48 hours. (With a later batch of seed, these times were extended to 48 and 72 hours.) On the first day of germination, which was taken arbitrarily as the day when the radicle reached 1–5 mm. in length, the seeds were transferred to pots of soil, and grown under artificial light with a 16-hour day.

For germination in the Cucurbitaceae, *vide* Crocker, Knight, and Roberts (1910); and in *Citrullus*, Hufford (1938). It is necessary only to add that, under our conditions, lateral roots begin to develop on the 4th day; the cotyledons emerge from the soil on about the 5th day and immediately begin to turn green. Their extension continues, at first rapidly then more slowly, up to about the 15th day, after which their area remains more or less constant at about 1·2 cm.^{2} The first leaf of the epicotyl expands on the 12th or 13th day, and from this period onwards epicotyledonary growth continues rapidly. In 5 weeks our plants had developed 10–12 leaves, and, at about the beginning of the 5th week, the cotyledons became yellow and were shed.

The anatomical changes do not concern us greatly. Even in the seed stage the tissue of the cotyledon is differentiated into palisade and spongy parenchyma, together with a small number (about 5) of provascular strands; transverse sections of the cotyledon on the 1st and 13th day of germination are illustrated in Fig. 1. Recognition of the period of maximum cell-extension is dealt with later in connexion with the changes in water content. It is, however, important to know whether the great increase in size of the cotyledon is due to rapid cell-division or the mere extension of existing cells. Cell division does undoubtedly occur during the development of the vascular bundles from the provascular strands and in the formation of the veinlet traces; but the number of cells so produced seemed likely to be negligible in comparison with the total number of mesophyll cells.

Investigation by the cell-counting technique of Brown and Rickless (1949) confirmed that this was in fact the case. We may therefore assume that growth of the cotyledon is due almost entirely to the extension of existing cells and not to continued cell-division. This is extremely convenient, since it implies that a 'per seed' estimate of urease content may be treated as if it were a 'per cell' estimate.

Urease distribution. Preliminary tests showed clearly that there is a very high concentration of urease in the cotyledons, a small quantity in the upper half of the hypocotyl, appreciably less in the lower half, less still in the roots, and none at all in the young epicotyl. Comparison of this result with those of Granick (1937, 1938) suggests that in *Citrullus* the urease activity is concentrated to an even greater extent in the cotyledons than it is in *Canavalia* or *Soja*. However, it must be remembered that Granick's observations depend very largely on his histochemical method, more sensitive than either the qualitative or quantitative methods used in this work, but unreliable as an estimate of quantity above a certain minimum.

Histochemical investigation shows that until about the 6th day urease is widely spread throughout the entire cotyledon. In the 2 or 3 days which follow the urease disappears with remarkable abruptness from all the cells except those of the bundle-sheaths: no gradual or orderly disappearance from any particular tissue could be observed.

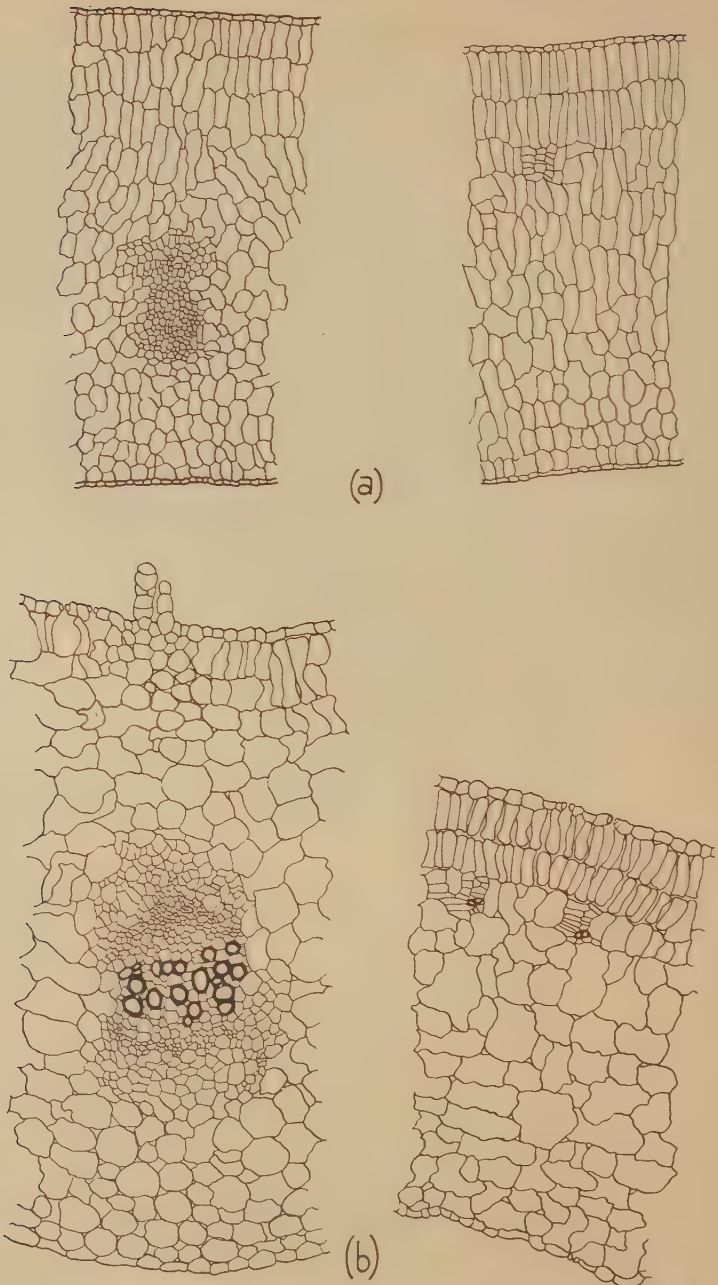


FIG. 1. Transverse sections of cotyledon at different ages: (*a*) 1 day, (*b*) 13 days. Midrib region on left, lamina on right.

In the hypocotyl and root, urease is detectable in a few cells near the vascular tissue, and isolated cells in the cortex or pith; no change was discernible in development. It is evident that the disappearance of urease from the cotyledons during their development must be regarded as the primary problem for investigation.

BIOCHEMICAL CHANGES DURING NORMAL DEVELOPMENT

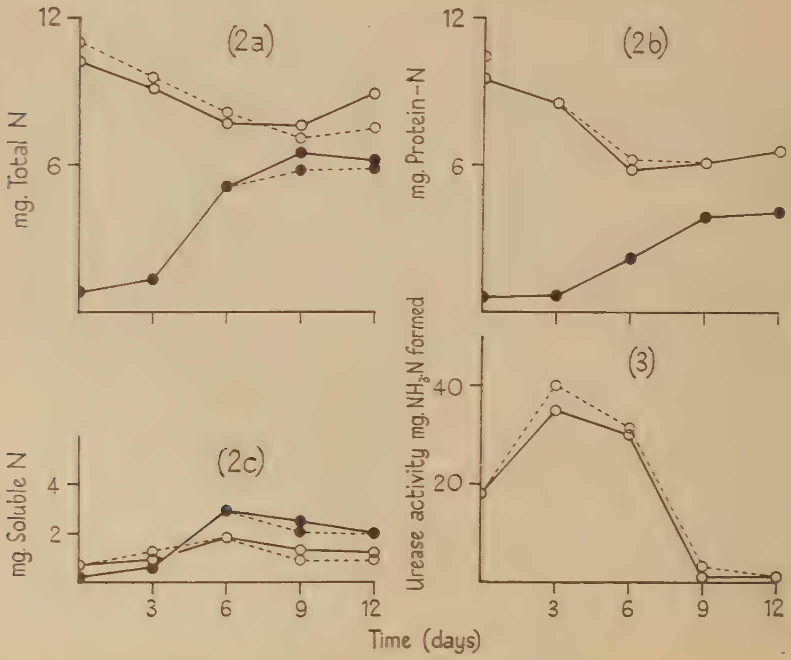
It was clearly essential first to ascertain more precisely the pattern of urease change during development, and to compare it with that of such other major biochemical changes as could conveniently be studied simultaneously. To this end we have investigated the changes (in the cotyledons, and in some cases the remainder of the plant also) in the following: urease activity; total, protein, soluble, and nitrate N; dry weight, water content and cell extension; invertase activity; chlorophyll content; and respiration. Every measurement was carried out on two replicate samples, each usually of four seedlings (the small sample reflects, not on the generosity of our suppliers, but on the limited space available under artificial light); and measurements were taken in most cases at 3-day intervals up to the 12th day of germination. Urease, the N-fractions, dry weight, and water content were all measured on the same group of plants; the cotyledons were detached at their bases with a razor blade and divided into two samples of 8 cotyledons, each sample having one cotyledon from each individual seedling. Fresh weight and dry weight were determined on one of these samples, and urease activity and the nitrogen fractions from the other. Separate groups of plants were used for the other measurements. Determinations on the remainder of the seedling (roots, hypocotyl, and epicotyl taken together) are labelled 'rest' in the diagrams which follow; it should perhaps be noted that recovery of the roots from the soil is never quite complete.

EXPERIMENTAL RESULTS

Total nitrogen (Fig. 2 *a*). The total nitrogen in the cotyledons decreases at first and then rises very slightly as the graph shows; total nitrogen in the rest of the seedling increases very markedly between the 3rd and 6th days and shows little change thereafter. It is evident that the cotyledonary store is drawn upon at first, but that after the 3rd day the rest of the seedling gains more nitrogen than the cotyledons lose. It will be recalled that lateral root formation begins on about the 4th day, and thenceforth the seedling is presumably gaining nitrogen from the soil. The rapid falling-off in the uptake of nitrogen by the rest of the seedling after the 6th day is likely to be due, in part at least, to the fact that the plants become pot-bound; it may well also be connected with the exhaustion of the available nitrogen supplies.

Protein nitrogen (Fig. 2 *b*). The changes more or less parallel those in total nitrogen. Protein breakdown in the cotyledons is complete by the 6th day. Constancy of protein content after the 9th day is no doubt due to the causes indicated at the end of the previous paragraph.

Total soluble nitrogen (Fig. 2*c*). In the plant as a whole soluble N is maximal on about the 6th day, when the external medium is displacing the cotyledons as a nitrogen source. The increase in cotyledonary soluble N between the 3rd and 6th days is clearly due to the breakdown of protein.



FIGS. 2 AND 3.

FIG. 2. Changes in (*a*) total N, (*b*) protein-N, (*c*) soluble N during germination. Results for one replicate are joined by continuous lines and for the other by broken lines. Where the broken line and second point are missing, both replicates agree within the accuracy of drawing. **Open circles, cotyledons: closed circles, rest of seedling.**

FIG. 3. Change in urease activity (2 replicates, each of 8 cotyledons) during germination.

Urease (Fig. 3). The urease activity shows an initial rise to the 3rd day, in contrast to the protein fraction, which is falling during this period. This rise might perhaps be considered to be paralleled by a small rise in soluble N however, urease decreases between the 3rd and 6th days, when soluble N is still rising. Between the 6th and 9th days, when there is no change in protein N, the urease practically disappears. It is evident that the patterns of urease and of protein change are totally different; and the suggestion (Williams, 1950) that urease is simply one component of the 'reserve' protein material of the seed cannot be maintained. (For histochemical observations see p. 141.)

Nitrate. Nitrate has been estimated by a comparison of a Kjeldahl determination in which reduced iron was used with a similar determination in which it was omitted, both determinations being carried out on aliquots from the same sample.

The results showed clearly that nitrate is present only in very small amount in the cotyledons, forming roughly 8 per cent. of the total N over the first 12 days of germination; whereas in the rest of the seedlings nitrate tends to accumulate, its proportion increasing from 10 per cent. of the total N on the 6th to 16 per cent. on the 12th day. Evidently *Citrullus* seedlings store nitrate, probably in the growing root system. As the present investigation is concerned primarily with the cotyledons, in which the amount of nitrate is negligible, nitrate reduction has not been carried out in the remaining experiments.

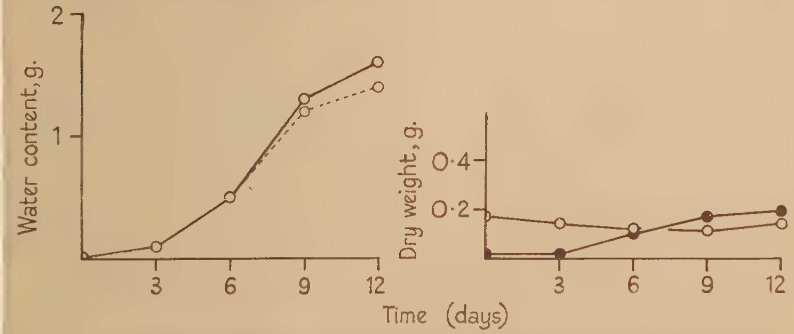


Fig. 4. Changes in dry weight and water content during germination (2 replicates, each of 8 cotyledons plotted using the same convention as Figs. 2 and 3). Open circles, cotyledons: closed circles, rest of seedling.

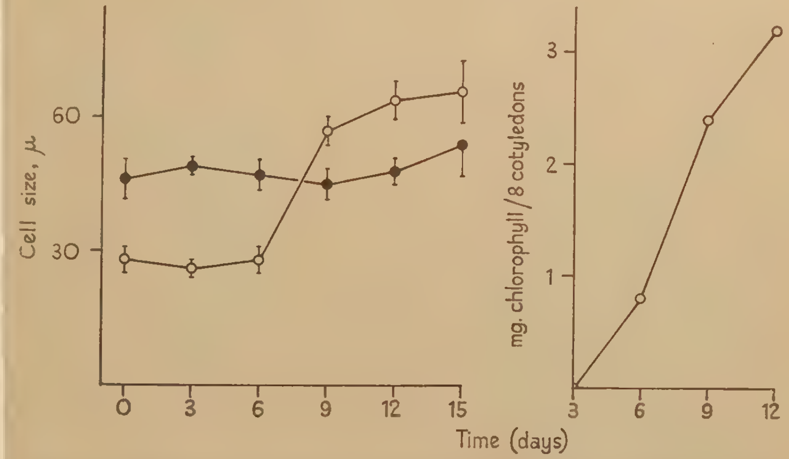


Fig. 5. Cell extension and chlorophyll content (of 8 cotyledons) during germination. Cell extension: open circles, lateral measurement: closed circles, vertical measurement.

Dry weight (Fig. 4 *a*). The slow decrease in dry weight of cotyledons is no doubt due to the translocation of nitrogen compounds to the rest of the seedling; the slight rise to synthesis of carbohydrate and possibly protein. The dry weight of the remainder of the seedling increases mainly between the 3rd and 9th days, during which period (under our experimental conditions) maximum root formation takes place. It will be recalled that by the 6th day the cotyledons are visibly green, and active photosynthesis is presumably in operation from this time.

Water content (Fig. 4 *b*). The water content of the cotyledons only is shown; data for the rest of the seedling have not been included owing to difficulties both of scale and of accurate measurement; but it may be noted in passing that as soon as lateral roots begin to form (after the 3rd day) there is an enormous increase in the water content of the rest of the seedling.

It is reasonable to postulate that the increase in water content closely parallels cell extension; but the actual process of extension can easily be measured approximately, and the results of such measurement are shown in Fig. 5 *a*. Every point on the graph represents an average of 20 measurements of cell diameters. The measurement of any particular cell was made in two directions, one parallel to the epidermis (indicated as 'lateral extension' on the graph), the other at right angles to this ('vertical extension'). No measurements were taken in the third (longitudinal) direction. Fiducial limits of ± twice the standard error of the means have been included, and it is clear that with the sample size here used the only significant change is that in lateral extension between the 6th and 9th days.

Chlorophyll (Fig. 5 *b*). Observation of the growing seedling shows that the cotyledons are not only expanding between the 6th and 9th days: they are becoming progressively greener. The change in chlorophyll content is shown in the figure, and it is clear that there is a sharp rise in content between the 6th and 9th days, paralleling the changes in water content and urease. The parallelism is, however, less exact in this case, since the chlorophyll continues to rise steeply for the next 3 days. It must be remembered that histochemical investigation showed that urease is retained in the bundle-sheaths long after it has disappeared from the mesophyll of the cotyledon; and that not only do the cells of the sheath appear to extend less than those of the palisade and spongy mesophyll, they also fail to develop chlorophyll. Some connexion between the development of chlorophyll and the disappearance of urease cannot be entirely ruled out.

With the size of sample available, the amount of carotenoid pigments was insufficient for accurate estimation. So far as could be ascertained, the final concentration was reached very quickly, and no changes comparable in magnitude with those exhibited by chlorophyll could be detected.

Invertase (Fig. 6). Brown, Reith, and Robinson (1952) noted a close connexion between invertase activity and cell extension in root-tips; as soon as extension ceased, invertase activity decreased. It is clear from the figure, however, that this relationship does not hold for our cotyledons; there is an increase to the 4th day, but no further change. The increase in invertase activity corresponds with that in urease activity in the early stages, but differs strikingly in the later stages in that it fails to show the characteristic fall. The maintenance of invertase activity at the same level after extension is over suggests that the metabolic behaviour during the extension phase of green photosynthetic organs such as cotyledons is (not surprisingly) different from that of root-tips. It is interesting to note in this connexion Granick's (1938) observation that the changes in the activities of protease and lipase in developing *Soja* followed a quite different course from the change in urease.

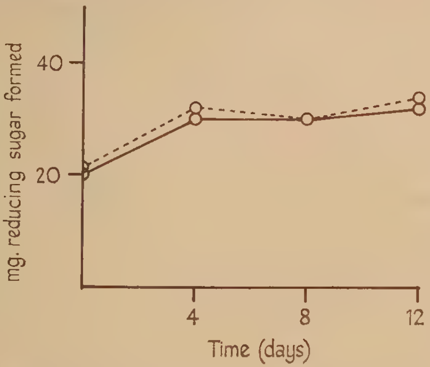


FIG. 6. Invertase activity of cotyledons during germination (2 replicates, each of 8 cotyledons). Results for one replicate are joined by continuous lines and for the second one by broken lines. Where the broken line and second point are missing both replicates agree within the accuracy of drawing.

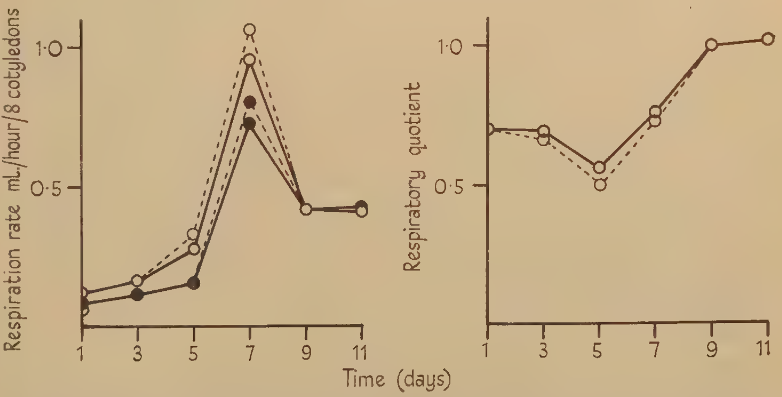


FIG. 7. Changes in respiration rate and respiratory quotient per 8 cotyledons during germination (2 replicates plotted using the same convention as for Fig. 6). (*a*) Respiration rate. Open circles, O_2 uptake; closed circles, CO_2 output. (*b*) Respiratory quotient.

Respiration (Fig. 7). The respiration of the cotyledons was followed for a period of 11 days after germination, readings being taken every 2nd day. Eight cotyledons were detached and put into Warburg flasks in darkness and O_2 and CO_2 changes followed for 5 hours; the rate of respiration was always high in the first hour—perhaps owing to the handling of the material (*vide*, e.g. Audus, 1935, 1939)—but by the end of this period the rate fell and soon reached a more or less steady level. The points in Fig. 7*a* represent values taken from this level phase.

The most striking features are, first, the remarkable peak at the 7th day (when cell extension is most active), and, secondly, the low R.Q. in the early stages (reaching unity only at the 9th day). An association between cell extension and respiration rate has been noted by Brown and Broadbent (1950); they found that in the growing zone of the pea root the rate of respiration per cell increased sharply during the period of extension, at the end of which it fell to a lower, constant, level. The respiratory quotient follows a course similar to that of *Ricinus* during germination (Stiles, 1936, pp. 126–7). Like those of *Ricinus*, *Citrullus* seeds contain a considerable quantity of oil – 53·67 per cent. of the dry weight of the kernel according to Langueld (1930); and the low R.Q. in the early stages of germination is presumably due to the oxidation of oil. The gradual increase in R.Q. from the 5th to the 9th day no doubt reflects the replacement, as a respiratory substrate, of oil by carbohydrate.

Implications. It is clear from the foregoing biochemical investigation that the change in urease activity of the cotyledons is virtually independent of changes in the major nitrogen fractions. Whatever the function of urease may be, it clearly cannot be considered simply as a 'reserve protein', since protein-N and urease-N show practically no parallelism of behaviour; and the total quantity of urease present, though very great by enzyme-concentration standards, is too small for it to act alone as a reserve food-substance.

There is, however, a very striking correlation between the characteristic fall in urease and the process of cell extension. As the cells undergo their main extension (6th to 9th days), their water content rises, they exhibit a marked respiratory peak and their urease activity falls. This suggests very strongly that changes in urease activity are no more than what may be called 'protoplasmic differentiation' (we owe this expression to Dr. R. Brown), i.e. changes in the enzyme complement of the protoplasmic structure as the cell develops into its mature form. The urease change perhaps represents no more than a stage in the differentiation of the mature protoplasm, but a stage which happens to show the special chemical properties which we associate with the *in vitro* activity of urease. Granick's (1938) conclusion that urease is a 'growth-bound enzyme' may reasonably be regarded as an early, and thus less explicit, statement of the same conception.

There remains the possibility of some connexion with the development of chlorophyll, which is taking place at the same time. This seems hardly likely—for example, urease is not detectable in the epicotyledonary leaves at any stage—but cannot be entirely excluded.

THE EFFECTS OF NUTRITIONAL STATUS

Experimental method. At this stage in the investigation it was decided to subject the plants to abnormal metabolic conditions, in the hope that the interdependence of urease and other changes might thereby be further clarified. A three-factor (2×2×2) experiment was carried out in sand culture as follows: *External nitrogen* (Knop's solution, either complete or with calcium nitrate replaced by the phosphate): Lack of external N might be expected to cause a heavier drain on the N reserves of the cotyledons. *External carbohydrate* (with or without added glucose 1 per cent.): This might be expected to affect breakdown of reserve protein—*vide* Chibnall (1938), and references cited therein. *Light* (a bank of 8 'daylight' fluorescent lamps, or an unlighted incubator at 25° C.): Darkness may be expected to interfere with chlorophyll formation and cell extension in the cotyledons.

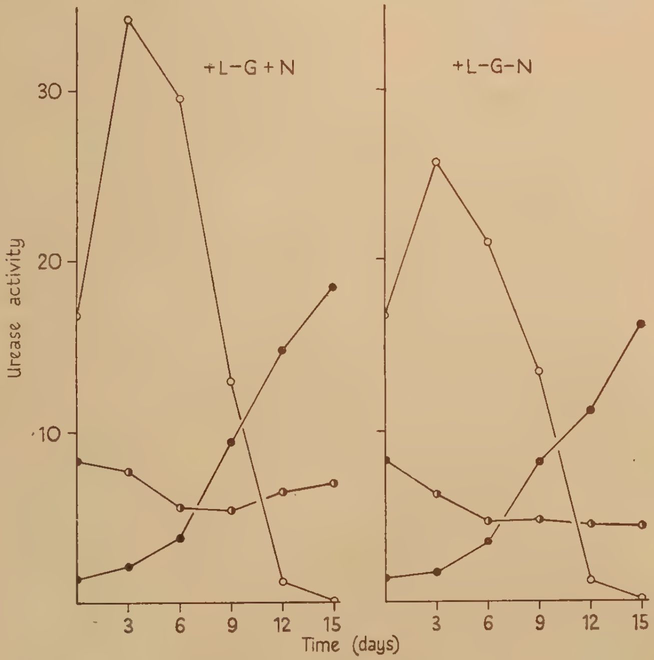


FIG. 8. Changes in urease activity (open circles), total N (half-closed circles) and fresh weight (closed circles) of 6 cotyledons under conditions (+L) (−G)(±N). Ordinate scales: urease and total N, 1 divn. = 1 mg. ammonia N: fresh weight, 1 divn. = 100 mg.

The pots were supplied with culture solution every 4th day, tap-water being added on the intervening days. In the experiments with added glucose, particularly those without nitrate in the light, contamination (fermentation with odour resembling butyric acid) was never completely avoided; this caused a slight reduction in growth of the affected seedlings. Fresh weight, total N, and urease activity were followed in the cotyledons (samples of 3 seedlings) over a period of 15 or 16 days.

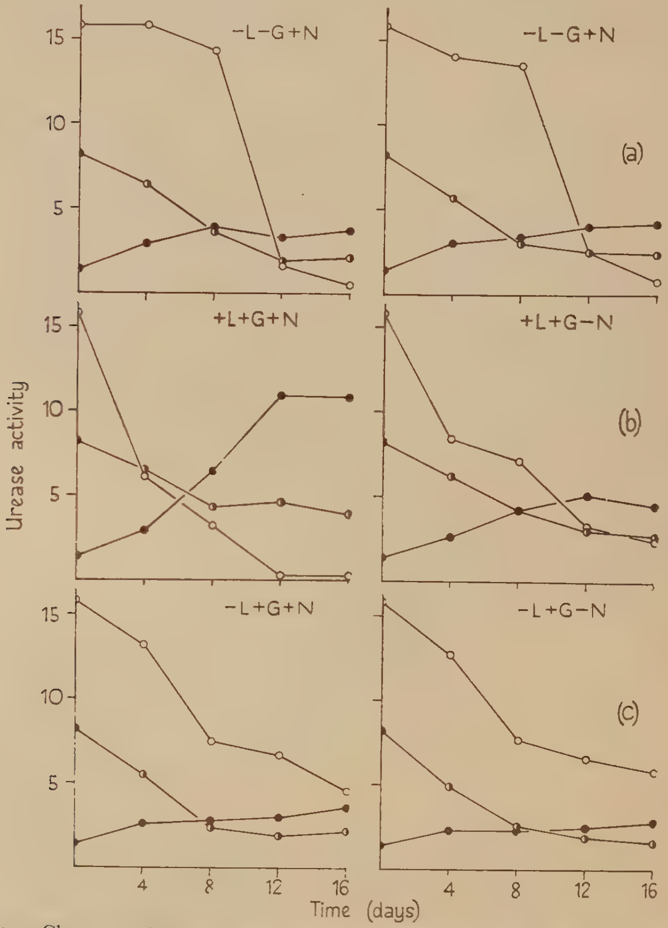


FIG. 9. Changes, as in Fig. 8, under the following conditions: (*a*) (−L)(−G)(±N); (*b*) (+L)(+G)(±N); (*c*) (−L)(+G)(±N).

Experimental results. The results are presented graphically as follows: Fig. 8: with light, without glucose (+L)(−G); Fig. 9 (*a*): without light, without glucose (−L)(−G); Fig. 9 (*b*): with light, with glucose (+L)(+G); Fig. 9 (*c*): without light, with glucose (−L)(+G). In each case the left-hand graph is (+N), the right-hand (−N).

Effect of nitrogen. Inspection shows that the (+N)(−N) pairs for each of the four combinations are almost identical. The total N in the (+N) combinations appears to show a slight tendency to increase slightly after about the 9th day of germination, but there is no difference in the early stages. The (+L)(+G) combination appears at first sight aberrant, since, under (−N) conditions, the urease appears to be disappearing more slowly; but it will be noted that fresh weight increase—and therefore extension—is also reduced; this is probably merely a result of the slight check in growth caused by contamination in the (+L)(+G)(−N) combination.

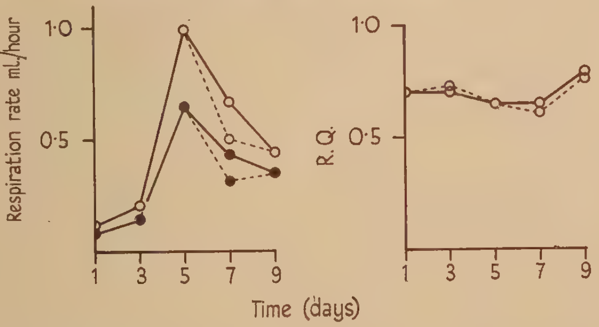


FIG. 10. Changes in respiration rate per 8 cotyledons and R.Q. during germination with added glucose (2 replicates). (*a*) Respiration rate. Open circles, O_2 uptake; closed circles, CO_2 output. (*b*) Respiratory quotient. Results for one replicate are joined by continuous lines, those for the second replicate by broken lines; where the broken line and second point are missing both replicates agree within the accuracy of the drawing.

Effect of darkness in absence of glucose. The most striking effects of etiolation are (i) suppression of the initial rise in urease activity, and (ii) the fact that, though extension is very greatly reduced, the fall in urease is delayed but not prevented. Total N falls more rapidly than it does in plants grown in light, probably because root-growth, and in consequence uptake of external N even when it is available, is reduced.

Effect of glucose. The effect on changes in total N is slight. In light there is rather more depletion of cotyledonary N than is the case in plants grown without glucose, but in darkness the amount of total N which disappears is practically the same whether glucose is supplied or not. There are, however, two striking positive effects: (i) glucose *always* greatly increases the speed of disappearance of urease, converting in the light the normal initial rise into a fall, and, in darkness, causing a more rapid fall than would otherwise occur; (ii) glucose in darkness, after the initial fall, delays the further disappearance of urease.

Implications. We may note in passing the not unexpected fact that the nitrogen-metabolism of the cotyledons, in the early stages of germination at least, is virtually independent of external nitrogen supply. The important effect is the early fall in urease brought about by glucose. This suggests that the ordinary progressive rise and fall of urease is an energy-requiring process, which can be accelerated by the provision of additional respiratory substrate in a readily available form. It is instructive to examine the respiratory behaviour of glucose-fed plants; Fig. 10 shows the O_2 uptake, CO_2 output, and R.Q. of cotyledons of the (+L)(+G)(+N) combination. It is precisely equivalent to the data for normally grown (+L)(−G)(+N) plants given in Fig. 7, and it is immediately evident that the addition of glucose has advanced the respiratory peak from the 7th day to the 5th. Brown and Sutcliffe (1950) have reported increased rates of respiration of root fragments as a result of the provision of sugar, and they suggest that sugar provides additional substrate for the cellulose synthesis (an energy-requiring process) necessarily involved in cell extension.

The 'substrate-sparing' properties of glucose are also interesting. The disappearance of urease in (−L)(−G)(+N)—which would not be expected if urease-disappearance is dependent on the onset of extension—might be due simply to the use of protein (including urease) as a respiratory substrate, the 'protoplasmic respiration' of F. F. Blackman; the addition of glucose might well be expected to 'protect' the protein constituents and thus delay the fall in urease. Furthermore, it will be seen from Fig. 10 that the addition of glucose delays the rise of the R.Q. to unity; this could similarly be attributed to a failure to oxidize fat to carbohydrate if free carbohydrate is available.

Urea as nitrogen source. If there is any connexion between cotyledonary urease and urea metabolism, the provision of urea as the sole nitrogen-source might be expected to produce interesting results. First, some abrupt change in the nitrogen-relations of the cotyledons might be expected, corresponding with the fall in urease; or the growth of the plant might be checked at this point and urea begin to accumulate. Secondly, the removal of the cotyledons before their main extension begins (if the plant can survive the operation) might be expected to cause the accumulation of urea at a still earlier stage. These possibilities have been tested experimentally.

Urea as nitrogen source. Plants were grown in sand cultures supplied with Knop's solution in which nitrate was replaced by urea in one set and by ammonia in the other (since urea is hydrolysed to ammonia, if urea is acceptable as a source of nitrogen, ammonium salts should be equally effective). The changes in urease, total N, and fresh weight of the cotyledons were observed over a period of 15 days after germination, six cotyledons being used as a sample. The results are represented in Fig. 11.

It is immediately evident that changes in total N are similar to those in nitrate-fed plants (cf. Fig. 8 (+N)); there appears to be a slightly heavier drain on N-reserves in the early stages of germination of urea-fed plants, but it is doubtful whether this is a real effect. Similarly, neither urea nor ammonia has any effect on the change of urease content with time. The cotyledons in both cases were slightly smaller than was usual in nitrate-fed plants, but in general the plants appeared perfectly healthy, and remained so for 5 weeks, when the experiment was discontinued.

Accumulation of urea. The urea content of seedlings (excluding cotyledons) was determined on the 6th, 9th, 12th, and 14th day after germination; a very small quantity (about 0·2 mg. per seedling) was present on the 6th day, a trace on the 9th, and none thereafter. It is quite clear that the disappearance of the bulk of the cotyledonary urease by the 9th day has not caused urea accumulation. This result, and the fact that the plants still grow well after 36 days (when the cotyledons have been lost), shows that cotyledonary urease has no necessary function in urea metabolism; evidently the small quantity of urease detectable in root and hypocotyl is perfectly adequate to handle all the urea needed to supply enough nitrogen for normal growth.

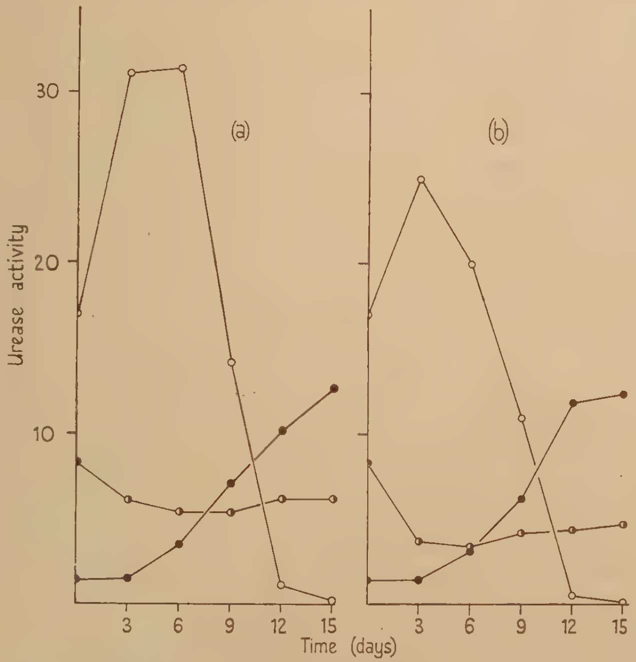


FIG. 11. Changes, as in Fig. 8, with (*a*) ammonia and (*b*) urea, as sole nitrogen-source.

Removal of cotyledons. Two sets of plants were grown, one with nitrate and one with urea; on the 6th day of germination the cotyledons of half of each set were removed with a sharp razor. The result was a severe check in growth from which the plants never recovered; whether this was solely due to loss of photosynthetic surface or whether it was partly caused by interference with root formation owing to a lack of some substance such as 'rhizocaline' (Went, 1938) we hazard no opinion. The urea-fed seedlings undoubtedly suffered the more, though this may merely mean that urea is a less suitable

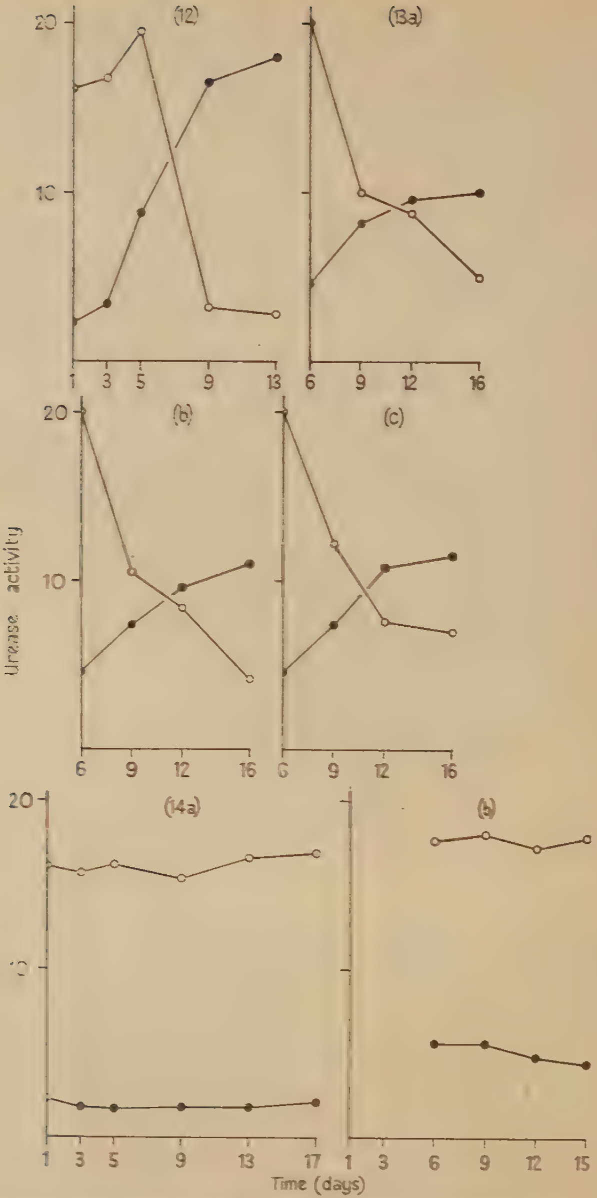


FIG. 12. Changes in urease activity (open circles) and fresh weight (closed circles) of 8 detached cotyledons. Ordinate scales as in Fig. 8. FIG. 13. Changes in urease activity (open circles) and fresh weight (closed circles) of 8 detached cotyledons in (a) water, (b) α-naphthylamine, and (c) adenine. Ordinate scales as in Fig. 8. FIG. 14. Changes in urease activity (open circles) and fresh weight (closed circles) in 8 cotyledons, detached on 1st or 6th day and sealed, left and right-hand graphs respectively. Ordinate scales as in Fig. 8.

source of nitrogen than is nitrate. The important point is, however, that investigation showed no abnormal accumulation of urea as a result of the removal of the cotyledons.

METABOLIC CHANGES IN DETACHED COTYLEDONS

If the disappearance of urease is related solely to the extension of the cotyledon, and not to any aspect of nitrogen metabolism in the plant as a whole, it may be expected to occur in cotyledons detached from the plant but otherwise free to expand. Three experiments have shown that this is in fact the case.

Experiment 1. Cotyledons were detached on the 1st day of germination, stood with their bases dipping into distilled water in small specimen tubes, and illuminated by a 1,000-watt incandescent lamp. At intervals, determinations were made of the fresh weight and urease-activity of a sample of 8 cotyledons. The results are shown in Fig. 12, from which it is evident that the pattern of urease change is identical with that in an attached cotyledon.

Experiment 2. In this case, working in a different laboratory, cotyledons were detached on the 6th day and allowed to expand under rather weak fluorescent light. Three sets were taken: one in water, the others in solutions (5 p.p.m.) of α-naphthylamine and adenine. It seemed possible from the observations of Kruyt and Veldstra (1947) on the effect of these substances on leaf growth in *Cosmos* that they might accelerate the extension of the cotyledons. Reference to Fig. 13, however, shows that this did not occur, and that urease change proceeded normally in each case.

Experiment 3. Cotyledons detached on either the 1st or the 6th day of germination were smeared with a thin film of vaseline and their bases sealed with wax. Loss or uptake of water was thus prevented; and it will be seen from Fig. 14 that, in cotyledons whose extension is thus prevented, but which do not suffer the drain on food-reserves attendant upon etiolation, urease activity does not fall. The treatment is drastic and, of course, also interferes with gas exchange; but the result is compatible with the view that urease-disappearance and cell extension are intimately linked.

CONCLUSION

We can now deal summarily with three of the four theories mentioned in the introduction. The theory of Armstrong and Horton (1912), that urease 'protects' the cells from cyanate produced by uncatalysed hydrolysis of urea, is incompatible with our observations that the epicotyl contains no detectable urease (p. 141), but that the plant will grow perfectly normally with urea as its sole nitrogen source after the cotyledons have fallen (p. 153). This argument is especially convincing in *Citrullus*, in which the urease appears to be concentrated in the cotyledons to a greater extent than is usual.

The theory that *cotyledonary* urease is concerned in the utilization of urea available as a nitrogen-source is also untenable and the theory (Williams, 1950) that urease is part of the 'reserve' protein of the seeds is incompatible with the absence of parallelism shown by protein-N and urease-N under a variety of conditions.

All the evidence has pointed to a close connexion between urease-activity and development (especially extension) of the cells of the cotyledon, and we may return with profit to a restatement in more explicit terms of Granick's (1938) theory that urease is a 'growth-bound enzyme'. The tendency to regard enzymes as separate entities, lying in the protoplasm as if they were no more than irrelevant inclusions, belongs to an earlier stage of plant physiology. It is becoming increasingly evident (*vide*, e.g., Robinson and Brown (1952)) that enzymes are intrinsic parts of the protoplasm, whose 'enzyme complement' thus changes as it differentiates into its mature form. All the evidence presented in this paper suggests that the urease present in such remarkable quantity in *Citrullus* cotyledons is no more than an indication of the state of the protoplasm attained by the cotyledons before their growth ceases in the developing seed. There seems little doubt that functional urease, capable of exercising its familiar *in vitro* properties, does occur in roots and hypocotyl; but there seems equally little doubt that cotyledonary urease is not involved in its activities. The unsolved problem which emerges from this investigation is the elucidation of the reasons why the protoplasm in different plants at similar stages of development (in this case the resting cotyledons) should exhibit such very different biochemical properties: why, in fact, development of protoplasmic structure in *Citrullus* cells goes through the 'urease' stage, whereas that in many other plants does not. This problem, however, must wait on a much greater knowledge of the structure of protoplasm than we at present possess.

ACKNOWLEDGEMENTS

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Studies in the Nitrogen Metabolism of Apple Fruits

THE CLIMACTERIC RISE IN RESPIRATION IN RELATION TO CHANGES IN THE EQUILIBRIUM BETWEEN PROTEIN SYNTHESIS AND BREAKDOWN

A. C. HULME

Ditton Laboratory, Department of Scientific and Industrial Research

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SUMMARY

1. A close parallelism in the drift of the rate of respiration and the protein-N/non-protein-N ratio is shown to occur both in apple fruits attached to the tree and when detached from the tree at various stages of development and stored for several months at 12° C.

2. In detached fruits the fall in respiration which occurs immediately (during the first 48 hours) after picking is only accompanied by a concomitant fall in net protein in very young fruits in which active cell division is taking place. Subsequently, in fruit of all ages when a climacteric rise in respiration occurs it is accompanied by a net increase in protein.

3. It is argued that the climacteric rise in respiration is a result of increase in the level of protein which will be expected to reduce the ATP/ADP ratio.

4. Over the climacteric period, although rate of respiration and net protein content both rise, R rises more rapidly than protein and, subsequently, falls at a faster rate than P. It is suggested that this may be due to the 'new' protein containing a higher proportion of enzyme(s) directly involved in respiration and leading, for example, to a reduction in the ATP/ADP ratio.

INTRODUCTION

THE modern trend in plant physiology is to concentrate investigation on the partial reactions coming under the heading of 'Respiration'. This is largely a result of the striking advance in knowledge of the biochemistry of glycolysis in yeast and muscle. Thomas (1952) has indeed said 'current researches bear on questions concerning the beginning of the process (respiration) and the integration of events. The outcome of these researches will determine whether or not the traditional term will survive as something more than a counter with which to reckon.' Again, when speaking of tissues such as fruits which contain protein in amounts which do not exhibit rapid fluctuations, and which, in the mature organ, remain constant (for a given variety) within relatively narrow limits, the suggestion that 'protein' and 'enzyme' are synonymous terms is no longer regarded as a daring speculation. Whether that 'protein' is always composed of the same enzyme systems in the same

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proportions, and whether the protein must be completely degraded and rebuilt for the 'synthesis' of a new enzyme are, however, still matters for speculation.

Although by means of new techniques it is possible to study partial reactions in relation to a definite fraction of the protein (enzyme) in certain selected tissues, these techniques cannot be satisfactorily applied to all tissues. Strongly acid tissues, such as apple fruits, containing low concentrations of total enzymes, present peculiar difficulties. Until these difficulties have been resolved little more can be done than to fall back on an examination of relationships between changes in respiration rate (CO_2 output), protein content, sugar and polysaccharide content, &c. Modern theories of energy-transfers through energy-rich phosphorylating systems provide a new basis for the interpretation of such primary data, even though such interpretations must involve a considerable degree of speculation. Such an approach has been made in recent years for the apple fruit by Robertson and Turner (1951) and Martin and Lewis (1952).

The present paper is designed to apply this method of approach to data obtained over a number of years for English varieties of apples during growth and in storage. A new hypothesis will be advanced in explanation of the rise in respiratory activity in fruits which has come to be known as the respiration climacteric (Kidd and West, 1930).

EXPERIMENTAL

Primary data and methods of analysis for Bramley's Seedling apples in 1936, 1938, and 1939 and for Early Victoria apples in 1939 have already been published (Hulme, 1950). Since similarities in behaviour occur from season to season, selections only of these data have been used in the present paper to illustrate the arguments developed. The source of the fruit and some of the respiration data for the Bramley's Seedlings for 1945 are also presented in this earlier paper. The methods of chemical analysis were the same for all the series. Since, however, the primary object of the 1945 work was to ascertain whether there is a general relationship between protein content and respiration rate in the first few hours after picking in young fruit when respiration rate is falling rapidly, a special procedure was required to ensure the rapid fixing of the tissue so that the measured protein content corresponded closely with the respiration rate at the time of 'fixing'. In previous studies, and in obtaining the data other than that for 1945 in the present paper, the method of 'fixing' the tissue, i.e. preventing further chemical change, at a given point in time was to transfer the fruit in closed tins to a store held at −20° C. Cessation of biochemical change becomes virtually complete when the tissue is frozen hard; under the conditions just mentioned this takes 1 to 2 hours. This period is sufficiently short when chemical analysis of the fruit is only being made at intervals of several days, but it is too long when, as in the instance of the Bramley's Seedling apples in 1945, it is desired to measure changes which may occur in the nitrogenous constituents over a time interval of 5 or 6 hours. Samples for analysis were, therefore, placed in tins of such a size that the fruit completely filled the tin and the tin was then either rapidly immersed in brine at −30° C. or in a box of solid CO_2 (approximately −80° C.). Calculations indicate that under these conditions the tissue freezes in from 10 to 20 minutes. All the data in the present paper refer to whole fruit (peel and pulp), excluding seeds, except in 1945 I where the embryo seeds were insufficiently differentiated for removal from the rest of the tissue.

All the storage and respiration data were obtained at 12° C.

EXPRESSION OF RESULTS

The primary data (Hulme, 1950) was expressed on a 'per apple' basis, the appropriate standard deviations being also presented. With the one exception mentioned below the statistical treatment will not be carried farther in the present paper since no exact balance-sheets will be drawn up; the arguments developed will be concerned only with considerations of relationships which might exist between one reaction type (e.g. sucrose synthesis) and another (e.g. protein synthesis) and how this might be expected to affect overall rate of CO_2 output. It is important in correlating change in respiration rate and net protein content *during the first few hours after gathering* (1945 data) to be certain that the fall in protein is significant. The magnitude of the significant difference ($P = 0{\cdot}05$) for protein and respiration rate will, therefore, be indicated here by vertical lines to the right of the graphs (Fig. 2).

Protein (*determined and expressed as protein-nitrogen*). For the purposes of the argument developed, we are here concerned throughout with change in the equilibrium between protein synthesis and protein breakdown. Changes in total nitrogen are small throughout the life of the apple fruit in relation to changes in carbohydrate. It is therefore convenient to express change in protein as a change of protein-N per cent. of total N. In fruit growing on the tree this does not show absolute net changes in protein, since total nitrogen is also changing during the major part of the growing period, but it does indicate whether the emphasis is on synthesis or degradation. For fruit on the tree protein-N will, therefore, be expressed both as per cent. of total N and as mg. per 100 g. fresh weight. In detached fruit, since no change in total nitrogen occurs, protein-N as per cent. total N does indicate absolute net change in protein.

Starch, sucrose, and reducing sugar. Concentration of carbohydrate is probably the determining factor in its rate of utilization. In general, therefore, starch sucrose and reducing sugar will be expressed as g. per 100 g. water. In detached fruits, stored in conditions which reduce water loss from the fruit to insignificant proportions, *change* per apple approximates very closely to *change* per 100 g. water or per 100 g. fresh weight. Since the primary data was expressed on a per apple basis, it is convenient to express carbohydrate on a per apple basis for detached fruits.

Respiration rate (CO_2-*output*). In conformity with other results published by the author, CO_2 output is expressed in mg. CO_2/100 g. fresh weight of tissue 100 hrs. In detached fruit, determinations have normally been carried out every 24 hours, and since continuous records of respiration rate of fruit almost invariably fall on smooth curves, the actual experimentally determined

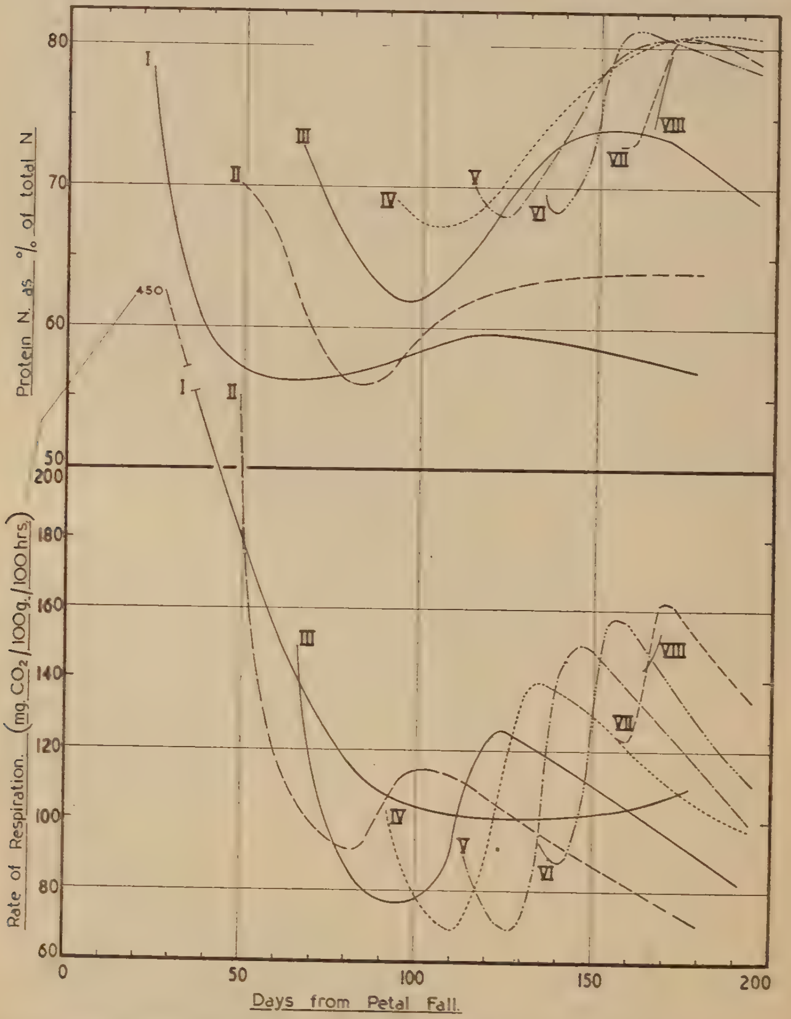

FIG. 1. Rate of respiration and protein content of Bramley's Seedling apples attached and detached from the tree at various stages of development. Season 1939.

points are omitted from the curves of CO_2-output to avoid unnecessary confusion on the graphs. Again to avoid confusion, the experimentally determined points for protein have also been omitted from the curves showing the overall trend in CO_2 output and net protein content (as a fraction of the total nitrogen) during growth and when detached from the tree throughout the growing season (Fig. 1).

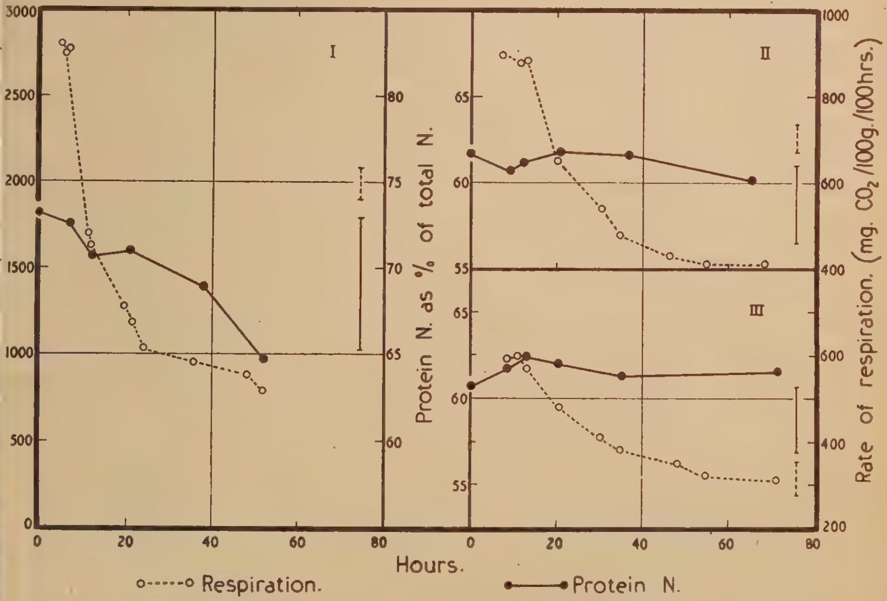

FIG. 2. Respiration rate and protein content of immature Bramley's Seedling apples during the first few hours after gathering. Season 1945. Picks I, II, and III as indicated. Respiration (– – – –); protein-N (——).

RESULTS

General. It has been clear for some time that there is a change in the balance between protein and non-protein nitrogen in detached mature apple fruits over the period of the respiration climacteric (Hulme, 1936). The close correspondence in time between change in protein : non-protein equilibrium and the onset of the climacteric both on the tree and in detached fruit *whenever it is picked* was only apparent after the drift in the two phenomena had been examined over several growing seasons. A typical series of results is shown in Fig. 1. At first sight this figure is somewhat confusing, but its object is to emphasize the similarity in drift between protein : non-protein equilibrium (P.E.) and respiration rate (*R*). Fruit was picked at the times indicated by the figures I, II, III, IV, &c., and several identical samples of fruit (see Hulme, 1950) were stored at 12° C. and their respiration rates determined. From time to time samples were frozen to −20° C. and subsequently analysed for protein content. In Fig. 1 the lines for *R* corresponding to the lines for P.E. will be seen by comparing the type of line (continuous, dotted, dot-dash-dot, &c.). From this figure it appears that, in general, not only is there a close correspondence in drift between P.E. and *R* over the climacteric, but also during the fall following detachment from the tree. To determine whether this parallelism commenced immediately after picking the 1945 data

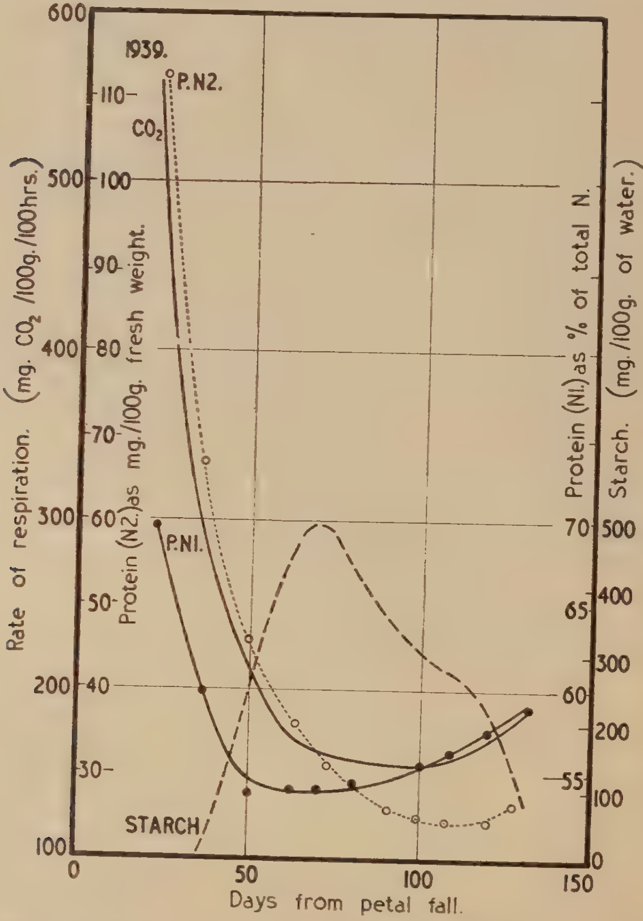

FIG. 3. Changes in rate of respiration, protein, and starch content of Early Victoria apples during development on the tree. Season 1939. Curve P.N1 represents protein-nitrogen as a per cent. of total nitrogen. Curve P.N2 represents protein-nitrogen as mg. 100 g. of fresh weight.

were obtained; respiration rate and protein content were determined in the first few hours after gathering. Results for fruit picked 18, 32, and 40 days from petal fall are shown in Fig. 2. Only in very young fruit is the initial fall in rate of respiration accompanied by an immediate fall in net-protein content. It appears probable, therefore, that the two phenomena, fall in *R* immediately after picking followed some time later (several days) by a fall in the P.E. and the rise in *R* accompanied by a rise in the P.E. which occurs over the climacteric rise in *R*, may be due to quite different metabolic processes. The present paper will be concerned chiefly with an examination of the second of these phenomena. It will be an advantage here to summarize certain apparently related events.

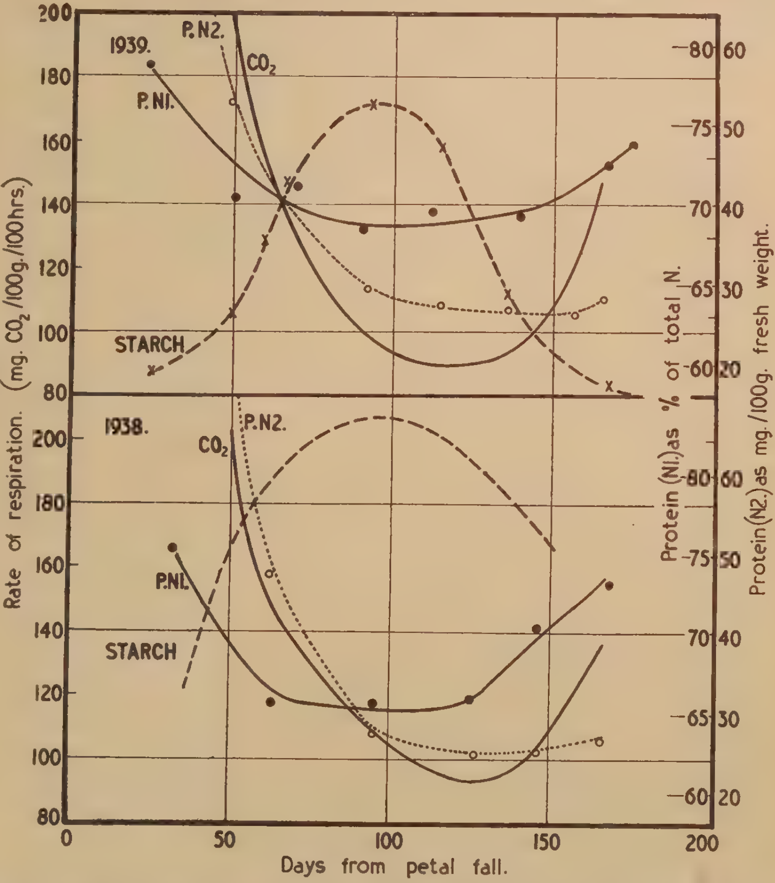

Fig. 4. Changes in rate of respiration, protein, and starch content of Bramley's Seedling apples during development on the tree. Seasons 1938 and 1939. The data for starch (1939) are plotted as mg./100 g. water, the value at maximum starch being ~1,800 mg. Starch (1938) is an approximate curve based on rough quantitative tests. (Hulme, 1950.)

A. *Changes occurring in fruit attached to the tree*

Changes in *R*, P.E., and carbohydrates taking place in fruit on the tree are shown for Early Victoria and Bramley's Seedling apples in Figs. 3, 4, and

5 respectively. Results for starch, *R*, and P.E. for 1938 as well as 1939 are included for Bramley's Seedling apples to show the similarity of events from season to season. The trend of events is as follows:

(1) *Bramley's Seedling apples.* Up to 60 to 70 days from petal fall (Figs. 4 and 5) there is a fall in net protein while starch and sucrose are beginning to rise; at the same time respiration rate is falling rapidly. From 70 to 130 days from petal-fall a balance is struck between protein synthesis and breakdown; the rate of starch synthesis falls off as the point of maximum starch is reached at 90 to 100 days, while sucrose continues to rise rapidly at first and then more slowly over this period (to 130 days); by the time the starch maximum is reached, fall in rate of respiration has practically ceased and remains approximately stationary between 100 and 130 days from petal-fall. From 130 days to the end of the observations starch falls to zero, net increase in sucrose falls off, and net increase in protein commences and gathers momentum; almost at the same time as this increased synthesis of protein occurs (1938 data) or slightly before (1939 data) CO_2 production increases rapidly towards the respiration climacteric.

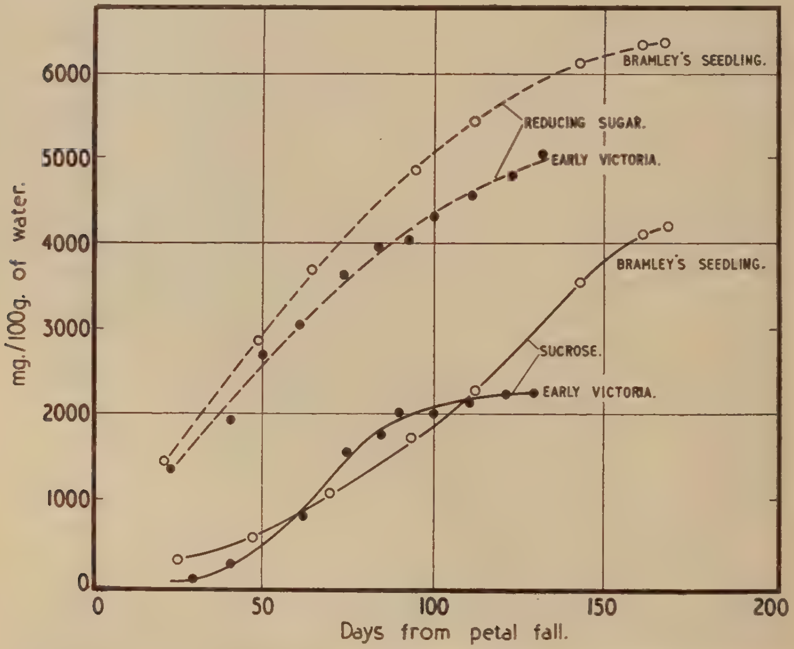

FIG. 5. Changes in concentration of reducing sugars and sucrose in Bramley's Seedling and Early Victoria apples during development on the tree. Season 1939.

(2) *Early Victoria apples* (Figs. 3 and 5). In this variety the sucrose to starch ratio is much higher and there is clear evidence that, during loss of starch, far more sucrose is being formed than could come from degradation of starch. Up to 50 to 55 days from petal-fall, net protein synthesis falls rapidly while starch and sucrose synthesis is only just commencing; respiration rate falls rapidly over this period. Between 50 and 70 days the protein level is maintained, starch and sucrose are increasing rapidly, and the fall in CO_2 production is checked. During the period of 70 to 105 days from petal-fall, respiration rate remains steady, starch falls rapidly and sucrose continues to increase but at a decreasing rate towards the end of the period; protein starts to rise at about 85 days. From 105 days onwards starch continues to fall almost to zero and net increase in sucrose practically ceases; net protein synthesis, however, increases and respiration rises to the climacteric peak.

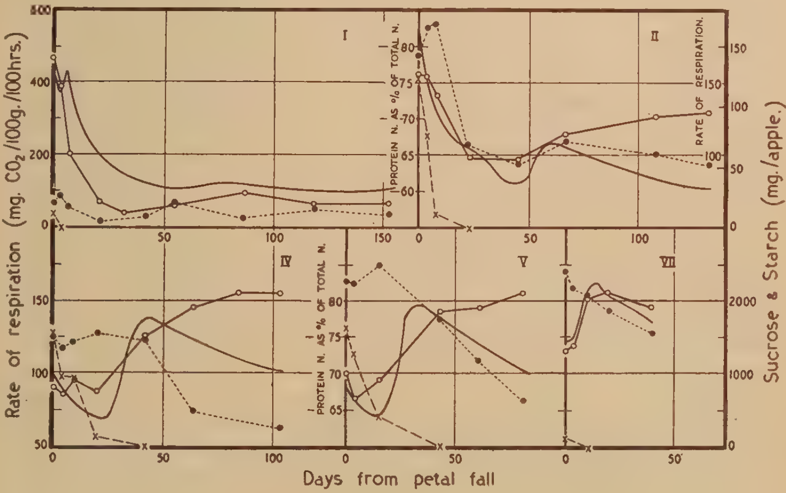

FIG. 6. Rate of respiration ——, protein content —○—○—, starch content –×––×–, and sucrose content --●----●-- of Bramley's Seedling apples after detachment from the tree at various stages of development. Roman numerals in the top right-hand corner of each graph indicate the pick.

B. *Changes occurring in detached fruit* (Bramley's Seedling apples only)

Although results are available for all 8 pickings in 1939 (see Fig. 1), the results obtained for Picks I, II, IV, V, and VII (Fig. 6) will serve to illustrate the trend of events. Where, as in Pick I, there is no real climacteric rise in *R* there is only a small increase in protein. In Pick II there is a definite climacteric, but its magnitude is small and the change in P.E. is also small in this fruit. Starch and sucrose are relatively low at picking in the fruit of both these series; the concentration of sucrose is less than 0·5 per cent. compared with 4 per cent. in mature fruit (Fig. 5). In early picks, starch, sucrose, protein, and *R* all fall almost immediately after picking. In later picks starch falls but sucrose is maintained and protein and *R* only fall slightly. If the fruit is gathered when the climacteric rise has already commenced, sucrose falls immediately but protein and *R* rise simultaneously (e.g. Fig. 6, Pick VII).

Before proceeding further it should be noted that Kidd, West, Griffiths, and Potter (1950) have provided evidence that no starch synthesis, only degradation (which does not involve phosphorylating systems), occurs in apples after detachment from the tree. In detached fruit, therefore, it would appear that energy-rich phosphate is concerned in two major processes—sucrose synthesis and protein synthesis. Whatever the mechanism for the conversion of starch to sucrose, and modern views regard both as involving phosphorylating systems (Albaum, 1952), it is clear from the data for the early gatherings (Fig. 6) that more sucrose is synthesized than could originate in starch.

DISCUSSION

During active cell division—up to $\sim$30 days from petal-fall (Tetley, 1931; Smith 1950)—the concentration of protein-N in Bramley's Seedling apples has been found, over a number of seasons, to lie between 260 and 100 mg. per 100 g. of fresh tissue. At about 30 days from petal-fall, protein-N forms more than 75 per cent. of the total N. By the time of maximum starch (Fig. 4) this protein content has fallen to about 30 mg. per 100 g. fresh weight and the P.E. ratio has shifted to 60 per cent. protein. That synthetic processes are fully stretched in protein synthesis during the period of active cell division is indicated by the rapid fall in protein which occurs *in the first few hours after detachment from the tree* at 18 days from petal-fall (Fig. 2, I). From 32 days onwards (Fig. 2, II and III) no such rapid fall occurs.

At the end of the cell-division phase in fruit on the tree, net protein synthesis falls rapidly (Figs. 3 and 4), the energy-rich phosphorylating systems involved in protein synthesis which are 'liberated' when synthesis decreases are more than sufficient for the starch and sucrose synthesis proceeding during this period. Whatever the mechanisms of protein synthesis, it now seems clear that it involves a high rate of phosphorus exchange (Wood, 1953). As argued by Robertson and Turner (1951), excess energy-rich phosphate will restrict respiration-rate by control of the *ADP ATP* ratio. There is, therefore, during this period a continuous fall in *R*. From 100 days from petal-fall in Bramley's Seedling apples (Fig. 4) and rather earlier in Early Victoria apples (Fig. 3), starch synthesis ceases and sucrose synthesis decreases (Fig. 5), but at the same time protein synthesis increases providing new acceptors for phosphate and this will decrease the *ATP ADP* ratio. Over this period, although net protein content is not increasing (Figs. 3 and 4, protein as per cent. fresh weight) overall synthesis is beginning to maintain the protein level (Figs. 1, 3, and 4, protein-N as a per cent. of total N). Finally, protein synthesis gathers momentum and outstrips breakdown and in consequence the respiration begins to rise to the climacteric. The full sequence of events over the respiration climacteric can only be followed in detached fruits, for the 'end of season fall' of fruit makes it impossible to do this 'on the tree'.

In fruit detached early in growth (Picks I and II, Fig. 6) carbohydrate reserves are very low (see also Fig. 5); synthetic processes (sucrose and protein) are relatively limited. In fruit of Pick I, a small net sucrose synthesis takes place at about the same time as a small rise in protein. This small increase in synthetic processes provides a limited supply of phosphate acceptors and there is a small rise in respiration but this is not comparable with the climacteric in more mature fruit. In Pick II fruit (49 days from petal-fall) net synthesis of protein is somewhat greater and a 'rudimentary' climacteric follows increased synthesis of protein and, to less extent, sucrose. Possibly in the young fruit energy-rich phosphate may be a limiting factor from the start. In all later picks until the development of the climacteric on the tree, a net increase in protein N of 10 to 15 per cent. of the total N occurs in association with a 'full' respiration climacteric (Picks IV, V, and VII, Fig. 6, serve to illustrate this). In the earlier picks the rise in protein *precedes* the climacteric rise in respiration, the two rises tending to occur simultaneously in mature fruit (Pick VII). In the earlier picks (e.g. IV and V) synthesis of sucrose is maintained for some time after picking and *before* the fall in sucrose gets under way, increased synthesis of protein commences providing an added sink for energy-rich phosphates. This leads to the rapid onset of the respiration climacteric. Subsequent fall in sucrose is more than offset by an increasing rate of protein synthesis and respiration continues to increase, the climacteric peak being reached as net increase in protein falls off. In later picks (VII, Fig. 6), fall in respiration after the climacteric peak is more rapid because in these fruits net protein synthesis is itself falling. Whether, in general, the fall in respiration after the peak is due entirely to a falling off in protein synthesis or whether it is due to a combination of this with a fall in sucrose (see later) may depend on whether the fall in sucrose is a result of a fall in net synthesis of sucrose or whether decrease represents breakdown only (not involving phosphorylating systems) and this is not known. Another possible explanation of the post-climacteric fall in respiration will be mentioned later.

The above argument does not, of course, explain *why* the increase in protein synthesis occurs in attached and detached fruits—it is suggested that this phenomenon and its cause is of more interest and importance than the respiration climacteric itself since the latter is but a result of the 'impulse' to increased protein synthesis at this period. The argument does, however, provide a tentative explanation of the observed association between net increase in protein and the climacteric rise in respiration.

Although change in the protein balance is associated with a similar change in rate of respiration, rate of respiration per unit protein (the R/P ratio) is not constant in detached fruit (this ratio is constant in fruit attached to the tree after the stage of cell division and up to the onset of the respiration climacteric—Hulme, 1939). In general the ratio falls on picking and rises to a new level over the climacteric. Typical examples of the drift in the *R/P* ratio are shown in Fig. 7—for Pick I and Pick IV. Where no climacteric occurs (Pick I) there is little deviation from a steady drift, but where there is a climacteric (e.g. Pick IV) the respiration per unit protein undergoes a large increase. Assuming that 'protein' represents total enzymes, the fall in the *R/P* ratio which follows detachment of the fruit from the tree may be due to a shortage of carbohydrate substrate. The rise in the *R/P* ratio to a higher value at the climacteric (i.e. when protein is also increasing) may be because the 'new' protein being synthesized contains a higher proportion of the enzymes directly involved in respiration.

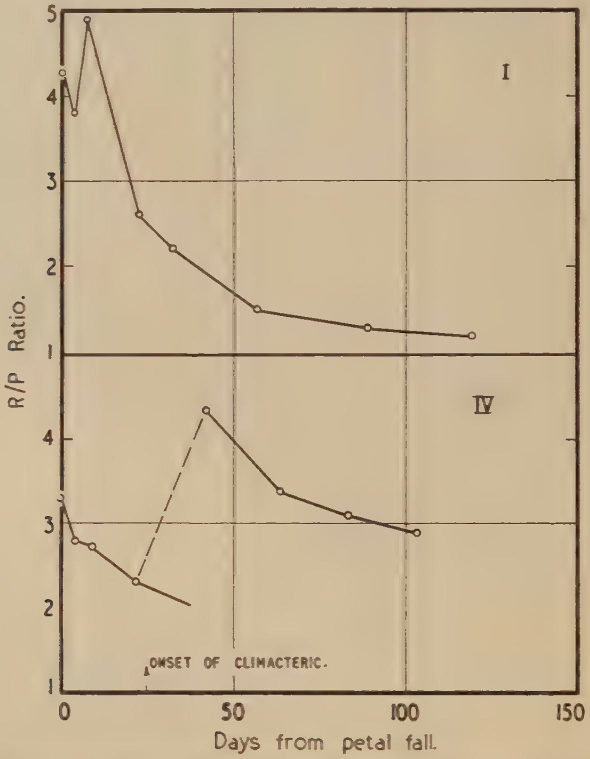

FIG. 7. Changes in the rate of respiration per unit of protein (the *R/P* ratio) in Bramley's Seedling after picking at various stages of development. Season 1939. Pick I: 15 June, 24 days from petal-fall; Pick IV: 22 Aug., 92 days from petal-fall.

This hypothesis provides an added reason for the increased respiration which is manifest as the climacteric and provides an explanation of the continuance of respiration at a higher, though falling, rate for some time after the climacteric peak. The steady fall from the peak which does occur in spite of only a small fall in net protein would then be due to the fall in sucrose and other respirable material. The post-climacteric sequence might then be considered as reproducing the fall in the R/P ratio after picking and as being a result of limiting substrate; the carbohydrate 'reservoir', starch, disappears about the time of the climacteric peak.

Finally, any theory connecting synthesis of protein with the climacteric rise in respiration in the apple must take cognizance of the fact that in apples of any age (at least greater than the '15 g. per apple' stage), when detached from the tree and subjected to low concentrations of ethylene, an immediate and simultaneous rise occurs both in net protein and in rate of CO_2 production (Hulme, 1948). Conversely when the respiration climacteric is delayed by storing apples in 10 per cent. CO_2 the rise in protein content is also delayed (Hulme, Kidd, and West, 1939). Treatment with ethylene does not appear to have any effect on sucrose and starch balance (Griffiths and Potter, 1950). On the basis of the theory outlined above an increase in protein synthesis would therefore be expected to be accompanied by an immediate increase in rate of respiration. It is suggested that the immediate effect of ethylene is to bring about a change in the balance between protein synthesis and protein breakdown.

These suggestions again, however, merely shift the emphasis of the 'climacteric phase' from respiration to protein balance; they do not throw light on the root cause of this striking phenomenon. Why should, for example, ethylene stimulate protein synthesis directly, if indeed it does so?

Much of the practical work involved in this paper was carried out by Messrs. W. Arthington, E. F. King, and L. S. C. Wooltorton.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and and Industrial Research.

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Note added in proof

In a recent paper Millerd, Bonner, and Biale (*Plant Physiol.* **28**, 521, July 1953) suggest that the onset of the respiration climacteric in avocado fruits may be a result of a decrease in the degree of the dependence of respiration oxidation on coupled phosphorylation. They do not mention the protein synthesis which occurs during the climacteric in apples or state whether such net synthesis of protein occurs in the avocado. In the light of their data these workers do, however, put forward the hypothesis that the uncoupling process, which they suggest is associated with ripening (i.e. the respiration climacteric), may be brought about by a soluble ATPase. This latter hypothesis is consistent with the suggestion put forward at the end of the present paper.

Oxalic Acid Metabolism in *Begonia semperflorens*

W. M. L. CROMBIE (*née* WOOD)

University of Southampton

Received 1 May 1953

SUMMARY

The primary accumulation of oxalate in the early seedling stages accompanies the synthesis of both protein and reserve carbohydrate. A further accumulation accompanies the normal growth of the young expanding leaf. Free oxalic acid accounts for most of the total oxalate content at the various stages.

The form in which nitrogen is supplied to the young seedling (ammonium nitrogen or nitrate nitrogen) markedly affects both growth and the amount of oxalate formed. More oxalate and poorer growth is found in plants grown on ammonium as a sole source of nitrogen.

In the mature leaf the oxalate content remains constant under a wide variety of different conditions.

No enzyme systems could be detected which might be active in the formation of oxalate.

It is concluded that oxalic acid is only formed during rapid growth in *Begonia*, and that once formed it takes little further part in metabolism.

OXALIC acid occupies an isolated position among the common plant acids. It occurs in considerable quantity in a few plants, for example *Begonia*, *Oxalis*, and rhubarb, and is also present in small amounts in a large number of different species. Unlike most of the other common organic acids, such as malic, citric, or isocitric, it is not known to take part in a respiratory cycle. It occurs generally in the 'bound' state, frequently in association with calcium ions—calcium oxalate then appears in a number of different crystalline forms (Pobeguin, 1943). In *Begonia* the acid is stated to be present predominantly in the free state, resulting in abnormally low pH values being recorded for the sap of this plant.

Various suggestions have been made about the function of oxalate in plant metabolism. Ruhland and Wetzel (1926, 1927) investigated the organic acids of rhubarb and of *Begonia semperflorens* and suggested that oxalic acid was formed by deamination of amino-acids with the simultaneous liberation of ammonia. Shibata (1932, 1933), working on *Begonia Evansiana*, agreed with this hypothesis. Later investigations on rhubarb by Bennet-Clark and Woodruff (1935), Allsopp (1937), and Pucher *et al.* (1937), showed conclusively that free ammonia did not accumulate and the amino-acid deamination theory was abandoned.

There is, however, evidence of a balance between oxalic acid formation and nitrogen metabolism. Ruhland and Wetzel showed in *Begonia semperflorens* that more total oxalate accumulated in plants cultured on nitrate

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nitrogen than in those cultured on ammonium nitrogen; similar results were obtained by Gilbert, Shear, and Gropp (1951) for tung.

The concentration of calcium also has an effect on oxalate accumulation as shown by Dunne (1932) for buckwheat and Olsen (1939) for a number of tree species. Other investigations have suggested a closer relationship to carbohydrate metabolism. In *Ricinus*, Scott (1941) found that etiolated seedlings contained less oxalate than normal seedlings, and that oxalate is formed during rapid growth in the light. As starch appeared, oxalate either disappeared or was not formed. A similar result was reported by Alexandrow and Timofeev (1926) for the flowers of *Stericularia*.

Vickery and Abrahams (1950) fed oxalate solutions to tobacco leaves and found a considerable uptake. They concluded from their results that oxalic acid is not an active metabolite. Zbinovsky and Burris (1952) using C^{14} labelled oxalic acid agreed with this result for tobacco, but found that in buckwheat some conversion of oxalic to other organic acids especially malic, succinic, and glycollic can occur.

EXPERIMENTAL METHODS

Begonia semperflorens var. 'Ball's red' was used throughout. Plants were grown from seed in shallow trays at a temperature of 65° F. ± 10° F. Fluorescent lighting was used to boost daylight giving a total of 16 hours light each day.

Analytical methods. The method of sampling and the procedures for total organic acids, titratable acid, reducing sugar, sucrose, starch, malic, citric, and isocitric acids were as recorded previously (Wood, 1952). Total nitrogen and soluble nitrogen were determined by the standard Kjeldahl method. Oxalic acid was determined by the method of Pucher *et al.* (1941). Lactic acid was estimated by the method of Barker and Summerson as described by Umbreit *et al.* (1949). Succinic acid was determined by the method of Krebs as described by Umbreit *et al.* (1949).

EXPERIMENTAL RESULTS

I. *Primary formation of oxalic acid in the young seedling*

Dry seeds of *Begonia* were analysed for organic acids and were found to contain only traces of oxalic acid at this stage. They were then germinated in soil and the complete plant analysed for organic acids, carbohydrates, and nitrogen fractions at intervals. The results are shown in Table I and in Fig. 1. The seeds contain an oil reserve as indicated by the figures for the ether extract. No attempt was made to analyse the separate components of this extract. Oxalic acid is quoted as total oxalate (i.e. 'free'+'bound' oxalate). Additional measurements were made of the 'free' oxalic acid and it was found that throughout this period of growth nearly all the oxalate was present as free oxalic acid. This result is confirmed by the figures for titratable acid.

It had been claimed by Ullrich (1926) that *Begonia* leaves contain small

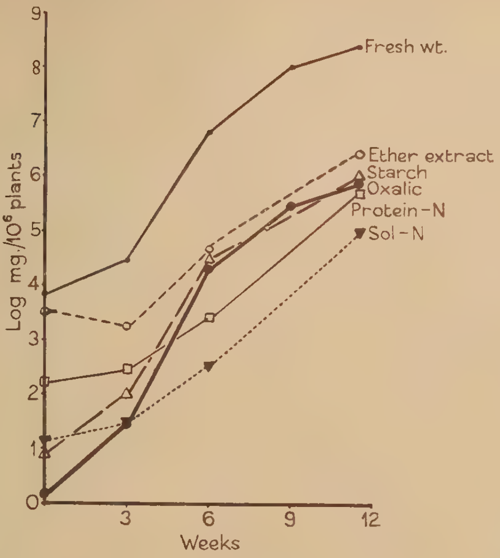

FIG. 1. *Begonia semperflorens* germinated in soil.

TABLE I

Begonia semperflorens: Germinated in soil

Results per 1,000 plants

	0 weeks	3 weeks	6 weeks	9 weeks	11½ weeks
Fresh weight	0·0074 g.	0·0524 g.	5.10 g.	105 g.	231·6 g.
Dry weight	0·0069 g.	0·0093 g.	0·237 g.	Not determined	10·83 g.
Protein N	0·172 mg.	0·283 mg.	7·734 mg.	,,	577·2 mg.
Soluble N	0·014 mg.	0·026 mg.	0·326 mg.	,,	98·8 mg.
Total acid	0·0004 m.eq.	0·0013 m.eq.	0·321 m.eq.	6·90 m.eq.	19·8 m.eq.
Titrat. acid	Not determined	0·0003 m.eq.	Not determined	Not determined	19·7 m.eq.
Oxalic	0·00003 m.eq.	0·0006 m.eq.	0·274 m.eq.	6·80 m.eq.	14·58 m.eq.
Malic	0·00002 m.eq.	Not determined	0·01 m.eq.	Not determined	Not determined
Citric	0·00009 m.eq.	0·0002 m.eq.	0·005 m.eq.	,,	,,
Isocitric	Absent	Not determined	0·02 m.eq.	,,	,,
Lactic	Absent	,,	Not determined	,,	Absent
Succinic	Absent	,,	,,	,,	Absent
Starch	0·0077 mg.	0·111 mg.	30·6 mg.	,,	1047 mg.
Reducing sugars	0·0093 mg.	0·052 mg.	1·478 mg.	97·5 mg.	132 mg.
Sucrose	0·067 mg.	0·058 mg.	1·10 mg.	43·7 mg.	99·4 mg.
Ether extract	2·954 mg.	1·896 mg.	180·7 mg.	Not determined	2800 mg.
R.Q.	Not determined	0·60	0·54	,,	1·01

amounts of lactic and succinic acids in addition to oxalic. The results given in Table I indicate that traces of malic, citric, and isocitric acids occur, but neither succinic nor lactic acids could be detected.

Formation of oxalic acid accompanies rapid growth and there is a close parallel with the production both of starch and of protein. Later stages of development were next considered by studying the accumulation of oxalate in the expanding green leaf. The weeks quoted refer to the time from germination. The leaves used were obvious at about 6 weeks. These results are shown in Table II. After the leaf has reached maturity and no further growth occurs, the formation of oxalic acid ceases.

TABLE II

The formation of oxalate in growing leaves

Results as mg. equivalents of acids per 100 g. F.Wt.
Results as mg. of carbohydrates and nitrogen per 100 g. F.Wt.

	20 weeks	24 weeks	27 weeks
Titrat. acid	10·3	23·1	20·0
Total acid	11·2	23·1	24·5
Oxalate	12·6	19·6	21·8
Reducing sugars . .	464·0	94·0	109·0
Total N	326·0	186·0	235·0
Soluble N . . .	70·0	4·0	39·0
Starch	186·0	314·0	432·0

II. *The effect of nitrogen supply on the primary formation of oxalate*

Ruhland and Wetzel claimed that the form in which nitrogen is supplied has a marked effect on the amount of oxalate produced by plants of *Begonia semperflorens*, but they gave no details on the age at which the treatments were given. In my experiments seedlings were grown in acid-washed sand and supplied with a complete culture solution containing nitrogen either as potassium nitrate or as ammonium sulphate. The culture solutions used were those of James (1936), and for the ammonium treatment, the potassium nitrate was replaced by an ammonium sulphate solution containing an equivalent amount of nitrogen. The plants did not grow as rapidly in these solutions as they did in soil, and the growth in the ammonium culture was particularly slow. The effects of the two treatments on various constituents are shown in Figs. 2 and 3.

Oxalate formation again occurs during rapid protein and starch synthesis, as in the results for seedlings grown in soil. These results can be recalculated in order to compare the effects of the different treatments on a basis of milligramme equivalents of oxalate per gramme of protein nitrogen. This gives the oxalate contents as: soil culture at 11½ weeks, 25·3 m.eq.; ammonium culture at 11 weeks, 41·6 m.eq.; nitrate culture at 12 weeks, 13·8 m.eq. This result is not in accord with that of Ruhland and Wetzel, who found the highest total oxalate content in nitrate cultivation, although they found the highest 'free' oxalate in the ammonium culture. Olsen (1939) also reports the highest 'free' oxalate in ammonium cultures for *Dianthus* and *Spinacia.* In the experiment above the oxalate is almost entirely present as the free

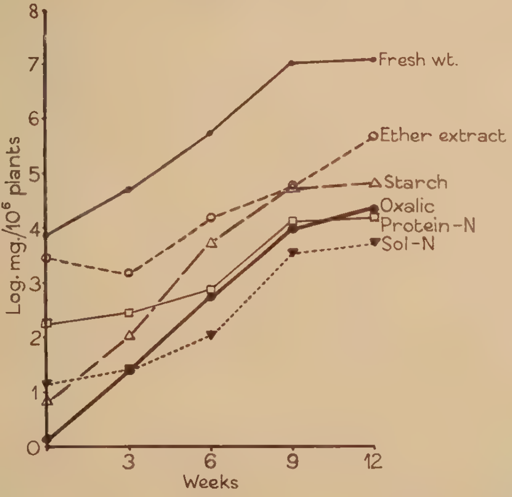

FIG. 2. *Begonia semperflorens* germinated in sand culture with nitrogen supplied as nitrate.

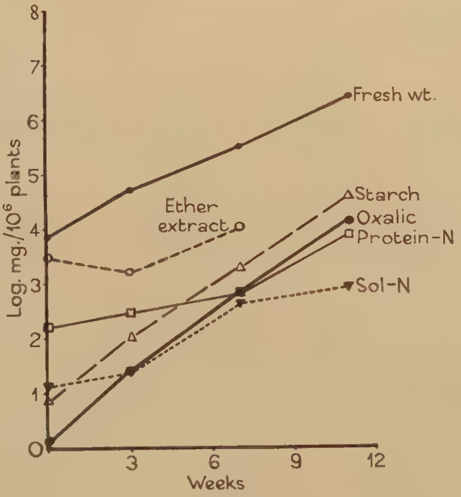

FIG. 3. *Begonia semperflorens* germinated in sand culture with nitrogen supplied as the ammonium ion.

acid, and this may account for the difference from the results of Ruhland and Wetzel.

III. *Metabolism of oxalate in the mature leaf*

(*a*) *Diurnal variation.* Ruhland and Wetzel reported a diurnal change in oxalic acid similar to that of malic and citric acids in the Crassulaceae, though of lower magnitude (—20 per cent. to 80 per cent. rise during the dark period). From my own experiments I have been unable to confirm this diurnal change: a typical result is presented in Table III.

TABLE III

Diurnal variation in Begonia semperflorens

Results expressed as in Table II

G.M.T.	Total acid	Titrat. acid	Total oxalate	Protein N	Sol. N	Reducing sugar	Starch
	m.eq.	m.eq.	m.eq.	mg.	mg.	mg.	mg.
8 p.m., 29.7.52 .	24·4	20·0	19·6	194	12	110	450
7 a.m., 30.7.52 .	25·0	24·0	20·4	159	39	101	420
3 p.m., 30.7.52 .	25·3	22·5	20·0	200	10	205	490

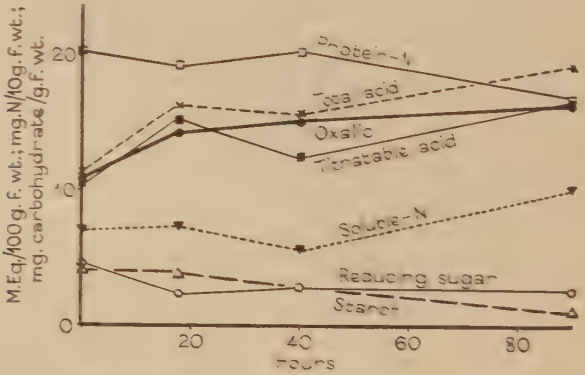

FIG. 4. Mature leaves of *Begonia semperflorens* darkened at 10° C.

Titratable acidity shows a fluctuation, but this is not due to any change in total oxalate or in total organic acids. It is possibly due to a component of the soluble nitrogen fraction which rises at the same time.

(*b*) *Formation of organic acids in the dark.* The accumulation of certain organic acids during darkness has been known for many years in the Crassulaceae and it has also been shown that more acid is formed at a low temperature than at a higher. *Begonia* leaves were therefore darkened at 25° C. and 10° C. in order to determine if these treatments had any effect on oxalate formation.

At 25° C. no significant change was found in titratable acid, total acid, or oxalic acid in up to 48 hours. The results of the experiment at 10° C. are shown in Fig. 4. A small increase in oxalate occurs, and carbohydrates are lost. Protein is converted to soluble nitrogenous compounds, and it can be seen again that the curve for titratable acidity follows the curve for soluble nitrogen closely.

This experiment was repeated in an atmosphere of 5 per cent. CO_2 in air, but the results were almost identical to those in air. No extra formation of oxalate occurred in the presence of CO_2, and no dark fixation of CO_2 could be demonstrated using a gas-analysis technique (Wood, 1952).

Heath (1950) reported a dark fixation of CO_2 by a related species *Begonia sanguinea*, and leaves of this plant were therefore darkened at 10° C. in a 5 per cent. CO_2 in air mixture. The results of this experiment are shown in Table IV. CO_2 fixation is confirmed, but was not accompanied by any significant changes in total acids, total oxalate, or titratable acidity.

TABLE IV

Darkening of Begonia sanguinea leaves at 10° C. in 5 per cent. CO_2 in air mixture

Results as in Table II

	0 hours	12 hours	36 hours
Total acid	24·1 m.eq.	22·5 m.eq.	20·0 m.eq.
Titrat. acid	18·1 m.eq.	18·0 m.eq.	19·9 m.eq.
Total oxalate	19·7 m.eq.	19·0 m.eq.	17·9 m.eq.
CO_2	−1·7 ml./hour	+0·4 ml./hour	

TABLE V

Effect of more prolonged darkening of Begonia

Results as in Table II

	Dark			Light
	2 days	10 days	12 days	19 days
Titrat. acid	8·84 m.eq.	8·6 m.eq.	6·9 m.eq.	12·2 m.eq.
Total acid	16·1 m.eq.	14·9 m.eq.	14·0 m.eq.	17·0 m.eq.
Oxalate	14·0 m.eq.	13·2 m.eq.	12·6 m.eq.	15·4 m.eq.
Reducing sugar	17·9 mg.	20·0 mg.	0 mg.	65·3 mg.
Starch	539 mg.	227 mg.	92 mg.	349 mg.
Protein N	141·6 mg.	80·2 mg.	90·0 mg.	131 mg. N
Soluble N	22·0 mg.	75·5 mg.	79·5 mg.	30·6 mg. N

The effect of more prolonged darkening was then investigated (Table V), and this is observed to have no significant effect on total oxalate, although marked changes occur in the nitrogenous fractions and in the carbohydrates. After nearly 2 weeks in the dark, such plants were replaced in the light. Further considerable changes occurred in the nitrogenous components and in the carbohydrates, but the oxalate remained almost unaltered.

When plants are left for 3 weeks or more in the dark, the older leaves start to die; it is found that such leaves still contain a large quantity of oxalate. At the same time young leaves start to develop, which are pale yellow in colour. These leaves have a low oxalate content, and their behaviour on transfer to normal days is shown in Fig. 5. The oxalate content increases slowly as in young leaves developing normally in the light (Table II).

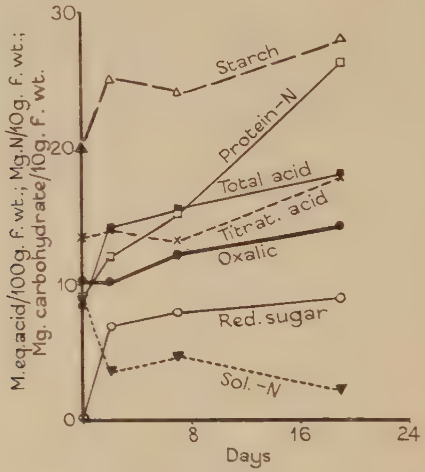

Fig. 5. Effect of transferring young leaves developed in the dark to normal days.

(*c*) *Feeding experiments.* Small complete plants were transferred from soil to sand at 20 weeks and after a further 2 weeks placed in darkness for 3 days in order to deplete the carbohydrate stores. After this they were watered daily for a total of 9 days with 10 ml. each per day of a 0·02 M. solution of the following compounds: glucose, pyruvic acid, citric acid, aspartic acid, glutamic acid, potassium nitrate, and ammonium sulphate —the pH being adjusted to 6 where necessary with potassium hydroxide. The results are shown in Table VI, from which it can be seen that none of these treatments has any effect on the oxalate content.

TABLE VI

Whole plants of Begonia semperflorens fed with different solutions in the dark for 9 days. Results expressed as in Table II

	Original sample (darkened 2 days)	Pyruvate	Glucose	Citrate	Aspartate	Glutamate	Nitrate	Ammon.	Water
Titrat. acid . .	8·8	8·6	9·1	6·2	8·0	7·2	8·1	8·0	6·9
Total acid . .	16·1	14·5	14·6	—	14·5	14·1	13·5	13·6	14·0
Oxalate . .	15·1	14·0	14·1	—	14·2	13·9	13·4	13·2	12·6
Reducing sugar .	17·9	20	6·1	—	6·7	38·6	19·2	0	24·1
Starch . . .	539	250	227	—	183	158	185	149	92
Protein N . .	114·6	81·5	80·2	—	102·1	127·6	90	106·1	85·5
Soluble N . .	22	48·5	50·5	—	63·9	68·4	49	70·9	49·5

Another type of feeding experiment was attempted in which mature leaves were placed with their petioles in solutions of 0·005 M. glycollic acid and 0·01 M. citric acid, both adjusted to pH 5·8 with sodium hydroxide. After 24 hours the leaves were analysed, but no change had occurred in the oxalate content.

In both the experiments just described the lack of penetration of the required compounds may explain the negative results. Finally the effect of glycollate and of citrate was tried on leaf slices. These were placed in 0·05 M. and 0·005 M. glycollate and 0·01 M. citrate solutions, all adjusted to pH 5·8 with sodium hydroxide. The effect of these treatments on respiration and on oxalate content was studied and compared with a water control. The 0·05 M. glycollate proved toxic. In 0·005 M. glycollate there was no effect either on respiration or on oxalate content. A similar result was obtained with the 0·01 M. citrate.

(*d*) *Experiments on leaf extracts.* The possibility remained that penetration was not occurring in the experiments just described and attempts were therefore made to extract the enzymes concerned in oxalate formation from the leaf tissues. Leaves were quickly crushed in a chilled mortar and the juice squeezed through three thicknesses of cheesecloth and rapidly adjusted to the pH required with strong potassium hydroxide. The extract was then centrifuged for 3 minutes at 1,500 r.p.m. in order to remove the larger cell fragments. A dark green juice remained which served as the crude enzyme extract. 1·5 ml. of juice was used in each experiment with 1 ml. of phosphate buffer in a Warbur flask. 0·5 ml. of the solution to be tested was placed in the side arm, and the centre well contained 0·2 ml. of KOH. The temperature was 25° C. The following organic acids were tested at pH 6·8 for their effect on oxygen intake by the juice: citric, malic, succinic, lactic, glycollic, formic, acetic, oxalic, and ascorbic. Malic, citric, succinic, lactic, oxalic, and ascorbic acids had no effect on oxygen intake, while a depressing effect on the small oxygen intake of the water control was shown by glycollic, acetic, and formic acids.

It has been shown by Kolesnikov (1940) for *Chlorella* and for barley leaves (1948) that these tissues contain an active glycollic acid oxidase. This α-hydroxy-acid oxidase has also been found in a wide variety of green tissues by Clagett, Tolbert, and Burris (1949*a* and *b*), and has been shown to oxidize glycollic to glyoxylic acid. The enzyme is only formed by green tissues in the light (Tolbert and Burris, 1950). Kenten and Mann (1952) have demonstrated in tobacco leaves the formation of oxalate from glycollic and glyoxylic acids. The oxidase enzymes concerned can in every case be extracted by the procedures used above and have pH optima of approximately 8. Zbinovsky and Burris (1952) using C^{14} labelled glycollic acid demonstrated the formation of C^{14} labelled oxalate in the buckwheat leaf.

Glycollic and lactic acids were tested on *Begonia* extract at pH 8, but no extra oxygen intake was found in any experiment. A test on the effect of glycollate and *l*-lactate on spinach-leaf extract at pH 8 showed a rapid oxygen intake and it was assumed that the method used in preparing the extract was satisfactory.

Begonia extract was shown to contain an active catechol oxidase both by Warburg experiments at pH 6 with added catechol and by the guiacum test.

DISCUSSION

From the results described there appears to be little doubt that oxalic acid is not an active metabolite in *Begonia semperflorens.* Its formation in the seedling is slow even during the most rapid phase of growth and protein synthesis. A large proportion of the total oxalate content is present at all stages as the free acid, giving the remarkably low pH values recorded for the cell-sap of this plant. The amount of oxalate formed is affected by the nitrogen nutrition, and this appears to be due to the effect of these treatments on general growth. The accumulation of oxalate under a number of different conditions parallels that of the formation of starch, suggesting that it may arise from an intermediate in photosynthesis. It has been shown by Shou *et al.* (1950) that glycollic acid is one of the first products of photosynthesis, and it has also been demonstrated by Tolbert *et al.* (1949, 1950), and Kenten and Mann (1952) that this acid can be converted into oxalic acid in green tissues. No evidence could be obtained for the existence of such a system in *Begonia semperflorens*, but as oxalate is formed only slowly in this plant, such a system might not be very active.

After its primary formation oxalate remains at a constant level in the mature, non-growing leaf. No evidence could be obtained of a diurnal variation in oxalate content, and even on prolonged darkening oxalate disappeared only very slowly and the leaves died while still containing a large amount.

I should like to thank Professor W. T. Williams for his helpful criticism of the script.

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Interactions in the Physiological Effects of Growth Substances on Plant Development

G. E. BLACKMAN AND R. C. ROBERTSON-CUNINGHAME

Department of Agriculture, University of Oxford

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SUMMARY

The interrelated effects of 3-indolylacetic acid and sodium 2 : 4-dichlorophenoxyacetate on the growth and development of *Lemna minor* and *Helianthus annuus* have been investigated by means of multifactorial experiments, involving three to five levels of each compound. A clonal population of *L. minor* was maintained under constant conditions of light (550 foot-candles) and temperature (25° C.) and the growth regulators were added to a phosphate-buffered culture solution (pH 5·1), which was changed every 2 days. In the *H. annuus* experiments the compounds, in aqueous solution, were placed by means of a micropipette on the leaves of young plants grown in a greenhouse or in the open.

Indolylacetic acid, at concentrations up to 25 p.p.m., increases the relative growth rate of *L. minor* on either a dry-weight or frond-area basis. Sodium dichlorophenoxyacetate at 0·025 p.p.m. has little effect on growth, but at and above concentrations of 0·2 p.p.m. growth is depressed. The ratio of frond area to total dry weight is increased by indolylacetic acid but is depressed by the phenoxyacetate. In the presence of indolylacetic acid, more particularly at the higher levels, the relative inhibition of growth caused by sodium dichlorophenoxyacetate is reduced.

When sunflowers are treated daily with indolylacetic acid (0, 6, 30, 150 μg./plant) or initially with sodium dichlorophenoxyacetate (0, 20, 100 μg./plant) in all possible combinations, then the relative growth rates of the treated leaves, the leaves above the treated leaves, the first internode, and the roots are depressed when each compound is applied alone. On the other hand, the growth rate of the hypocotyl is increased by both growth regulators, while for the second internode indolylacetic acid decreases the growth rate but the phenoxyacetate at the lower level accelerates it. The combined action of the two compounds on the different parts tends to be cumulative, save for the hypocotyl, where the effect of indolylacetic acid is dependent on the level of the phenoxyacetate.

It is concluded that these results cannot be explained on the concept of wholly additive effects. There is evidence that the compounds can mutually interfere in their actions and it would seem that more than one mechanism is involved. Many of the results can be satisfactorily interpreted on the basis that the two compounds act on a common system and that they compete with one another, but the results are not compatible with the postulate that the dichlorophenoxyacetic acid directly inactivates indolylacetic acid.

INTRODUCTION

In recent years the agricultural importance of a number of substituted phenoxyacetic acids has greatly stimulated research into the physiological effects of

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synthetic and naturally occurring growth regulators. Numerous papers covering the several facets of the general problem have been published, and from a number of viewpoints these have been reviewed by Larsen (1951), Skoog (1951), Blackman, Templeman, and Halliday (1951), Bonner and Bandurski (1952), and Van Overbeek (1952). Although the physiological and biochemical characteristics of 2 : 4-dichlorophenoxyacetic acid and 3-indolylacetic acid have frequently been contrasted and compared, few direct comparisons of the effects of these two compounds alone or in combination had been made at the time when the present experiments were started early in 1951. Went (1948), employing the pea test, had briefly reported that both indolylacetic acid and 2 : 4-dichlorophenoxyacetic acid have the same basic response. At relatively high concentrations, mixtures exert an additive effect, but at low concentrations the reactions caused by mixtures are greater than those which would be expected from the responses brought about by the individual compounds. Manil and Straszewska (1950), working with *Helianthus annuus*, investigated the influence of the same two compounds on the growth of tumours induced by *Phytomonas tumefaciens*. They recorded that indolylacetic acid stimulated tumour formation to a greater degree than dichlorophenoxyacetic acid, but concluded that in mixtures the effects were not additive since the volume of the tumours were intermediate in size to those produced by the individual compounds.

In neither of these papers was there any statistical evidence of the significance of the observed effects or interactions, and it seemed desirable that a more precise study should be made of the possible interrelationships between the two compounds, more particularly in their differential effects on the growth of intact plants. As a first approach experiments were carried out with the relatively simple higher plant *Lemna minor*, which has the several advantages that it can be readily grown as a clonal population in water culture under constant conditions of light intensity and temperature. Subsequently a further series of experiments were undertaken with *Helianthus annuus*, grown in pots in the greenhouse or the open, and in this instance a detailed study of the changes induced in the root, stem, and leaves was carried out by the methods of growth analysis.

EXPERIMENTAL METHODS

The difficulties of an accurate assessment of the relative biological activities of even allied compounds have already been stressed (Blackman, 1952). Moreover, it has rarely been appreciated that the critical evaluation of possible interactions demands careful attention to the design of the experiment. In the present investigation a simultaneous comparison has been made of three to five levels of each compound in all possible combinations, with a maximum of twenty treatments, while the replication has varied from two to fivefold.

Experiments with Lemna minor. In October 1949 a single frond selection was made from a clonal stock of *L. minor* which had been grown under continuous illumination in an inorganic culture medium for several months. The culture solution to which the growth regulators were added was a modified form of one of the solutions which had been found satisfactory by Steinberg (1941) and contained per litre the following salts: KNO_3, 0·35 g.; KH_2PO_4, 0·80 g.; $MgSO_4.7H_2O$, 0·1 g., together with the elements iron, zinc, manganese, molybdenum, gallium, calcium, and boron in amounts equivalent respectively to 0·15, 0·04, 0·04, 0·02, 0·02, 5·0, and 0·02 mg./l. The main modification of the Steinberg solution was to quadruple the phosphate content and so increase the buffering capacity, since it had been established that changes in the pH greatly affected the activity of weak acids, like dichlorophenoxyacetic acid, to *L. minor* (Blackman and Robertson-Cuninghame, 1953).

After each batch of the appropriate culture solution had been made up, the pH was measured with a glass electrode and the level adjusted with either potassium hydroxide or hydrochloric acid to pH 5·1, since other experiments had shown that for the particular conditions this pH was optimal. The plants of *L. minor* were grown in 250 ml. Hysil beakers in which the solutions were changed at intervals of 2 days; to do this the fronds were lifted out of each beaker on a perspex spatula, rinsed in distilled water to free them from algae, and placed in fresh solution. With this frequent manipulation the changes in pH over the 48 hours rarely exceeded 0·2 units, while undue algal contamination was prevented.

The initial number of fronds placed in the beaker was kept small (10–25 fronds) so that in the fastest-growing cultures the fronds did not become overcrowded in a single beaker. Care was taken to ensure that the proportion of plants with different frond numbers was initially of the same order in each beaker.

For the experiments the beakers were placed in three water-baths (4 × 1 ft.) with an interconnected water-circulatory system to minimize temperature-differences between baths, and the temperature was controlled by thermostats at $25 \pm 0 \cdot 5^\circ$ C. The plants were continuously illuminated with 80-watt Osram daylight fluorescent tubes attached to reflectors made from aluminium sheeting and arranged in such a way that the height above the bath could be adjusted so that it received a constant intensity of 550 foot-candles. Since Stoyle and Jones-Thomas (1950) had found that with this type of lamp the light intensity between 100 and 4,000 hours shows only a very slow decline, the light intensity was checked at weekly intervals by means of an Evans selenium photo-electric cell covered with an opal glass screen. The photo-electric cell was in turn standardized against a 1,000-watt A1 Osram projector lamp, which had been calibrated to within ±2 per cent. of N.P.L. standards.

At the beginning of each experiment, after the batches of fronds had been counted, beakers were selected at random from the various treatments and the appropriate number of beakers withdrawn to determine both the initial dry weight and total frond area. For the estimation of dry weight the general method of Ashby, Bolas, and Henderson (1928) was followed. The samples, after being rinsed in distilled water, were placed in weighing-bottles and dried for not less than 90 minutes over silica gel at 50° C. at a pressure of 1 cm. of mercury. The accuracy of weighing was ±0·2 mg.

The technique developed for the measurement of area resembled that of Gorham (1941) and involved the use of a Leica camera and a Stewartry copier. The fronds, together with a celluloid disk on which was etched a circle of known area, were placed in a beaker of distilled water which was illuminated from below. The lens of the camera was attached to the copier, which was clamped at a distance of 13 in. above the fronds, and the image of the fronds focused on the ground-glass screen of the copier. The camera was then substituted for the screen and a photograph taken. The developed negatives were projected through an enlarger on to paper placed on a sheet of glass and the distance adjusted until a fourfold magnification of the circle on the celluloid disk was attained. A tracing of the outline of each frond was then made and the area determined subsequently with a planimeter.

At the end of the experiment the dry weights and areas were again determined in the same way. The results have been expressed as the rate of change during the experimental period using the conventional formula for relative growth rate (Williams, 1946).

Experiments with Helianthus annuus. Seeds of the semi-dwarf variety 'Pole Star' (Blackman, 1946) were planted in a sieved mixture of equal parts soil and sand, contained in either glazed earthenware pots or aluminium pots of 10-in. diameter. To ensure a high level of nutrient supply 2 g. of a complete commercial fertilizer were added per pot. Approximately 15 seeds were planted and after emergence the seedlings were singled to leave 4 plants per pot. The pots were left in the open, but for the application of the growth regulators they were transferred to a greenhouse.

At the start of an experiment the pots were arranged in blocks such that there was a minimum variation within blocks. Pots selected at random were then taken to determine the initial weights. The shoot was cut off at soil level and immediately again separated into the hypocotyl, cotyledons, first and second internode, and first and second pair of leaves, which included the apex. These parts, together with the roots, were then dried separately at 100° C. for 24 hours.

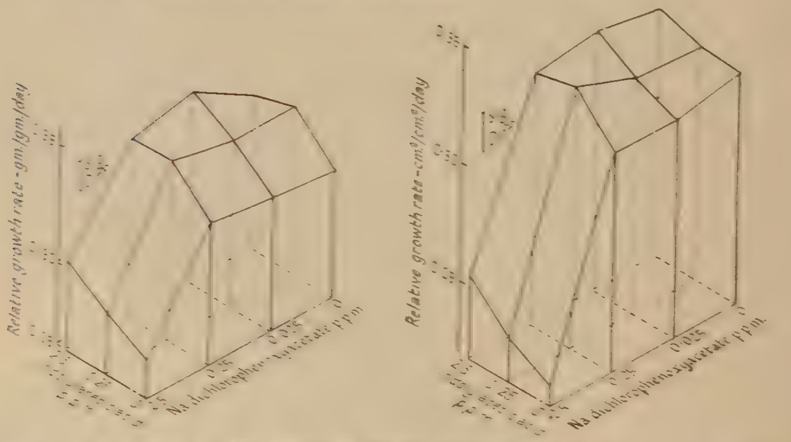
To collect the roots the soil from each pot was carefully washed through a sieve on which the roots were retained. Some loss of the smaller roots could not be avoided, but on the other hand a small amount of soil was retained by the roots, which on the basis of silica analyses amounted to less than 5 per cent. by weight. The same procedures were adopted at the end of the experiment and the relative growth rates over the experimental period of each part calculated.

The two growth regulators were applied in aqueous solution to the first pair of leaves by means of an 'Agla' micrometer syringe. This syringe is claimed to deliver droplets down to 1 μl. ± 0·05 μl.

EXPERIMENTAL RESULTS

The interrelated effects of 3-indolylacetic acid and sodium 2:4-dichlorophenoxyacetate on the growth of Lemna minor

In the first experiment three levels of indolylacetic acid (0, 0·25, and 2·5 p.p.m.) were combined factorially with four levels of sodium dichlorophenoxyacetate (0, 0·025, 0·25, and 2·5 p.p.m.). The replication was threefold and the experimental period was 7 days.



Figs. 1 and 2. Expt. 1. The effects of various combinations of sodium 2 : 4-dichlorophenoxyacetate and 3-indolylacetic acid on the relative growth rate of Lemna minor. Fig. 1 on weight, Fig. 2 on area basis.

At the lowest level of the phenoxyacetic acid growth was normal, but at 0·25 p.p.m. slight epinastic effects were observed, while at 2·5 p.p.m. the epinasty was more pronounced, root development was arrested, and the surfaces of the fronds became rugose. A concentration of 0·25 p.p.m. of indolylacetic acid, either alone or in combination with the two lower concentrations of the dichloro compound, caused a slight downward arching of the fronds giving a 'humped' appearance. This response was similar to, but not identical with, that produced by the dichlorophenoxyacetic acid alone. At the highest level of sodium dichlorophenoxyacetate the effects of indolylacetic acid at 0·25 p.p.m. were masked. On the other hand, a further increase in the concentration of indolylacetic acid caused a more pronounced 'humping' of the fronds and they assumed a darker green colour.

The changes in weight and area can be most appropriately expressed in terms of the relative growth rates of either weight or area, and the results are set out in Figs. 1 and 2. Statistical analysis of the growth rates showed that there was a highly significant variation between concentrations for both compounds ($P < 0·001$) but that the interaction between the two compounds was not significant.

In the presence of indolylacetic acid an increase in the concentration of phenoxyacetate from 0 to 0·25 p.p.m. has, in general, little effect, though on a weight basis there is for the highest level of indolylacetic acid a just significant increase in the relative growth rate. When the concentration of sodium dichlorophenoxyacetate is increased from 0 to 0·025 p.p.m. a corresponding increase on an area basis is apparent, but in this instance the differences are not significant. However, as the concentration of the phenoxyacetate is raised from 0·25 to 2·5 p.p.m. the relative growth rate on either an area or a weight basis is greatly depressed, the degree of depression being greater for area than for weight.

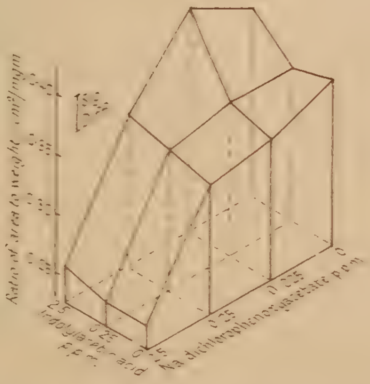


Fig. 3. Expt. 1. The effects of various combinations of sodium 2 : 4-dichlorophenoxyacetate and 3-indolylacetic acid on the ratio of frond area to total plant weight in *Lemna minor*.

At all levels of sodium dichlorophenoxyacetate an increase in the concentration of indolylacetic acid brings about an increase in growth rates, more particularly for changes in area. The fact that phenoxyacetic acid depressed and indolylacetic acid stimulated the change in area to a greater extent than the change in weight suggested that changes in the ratio of area to weight had taken place. Accordingly these ratios were calculated, and from the statistical analysis it appeared that a highly significant variation could be attributed to a concentration effect for both compounds, but the interaction was not significant. The effects are shown in Fig. 3, and once again the trends for the two substances are opposed. Increasing the concentration of indolylacetic acid causes some slight increase in the ratio, but the increase is only significant at the 0·025 p.p.m. level of the other compound. On the other hand, at all levels of indolylacetic acid increasing the concentration of the phenoxyacetate from 0·025 to 2·5 p.p.m. diminishes the ratio of area to weight.

Since in the first experiment the highest level of indolylacetic acid did not reduce the relative growth rate, a further similar multifactorial experiment was carried out but using higher concentrations of 0·02, 1, 5, and 25 p.p.m., together with four levels of sodium dichlorophenoxyacetate (0, 0·2, 1, and 5 p.p.m.).

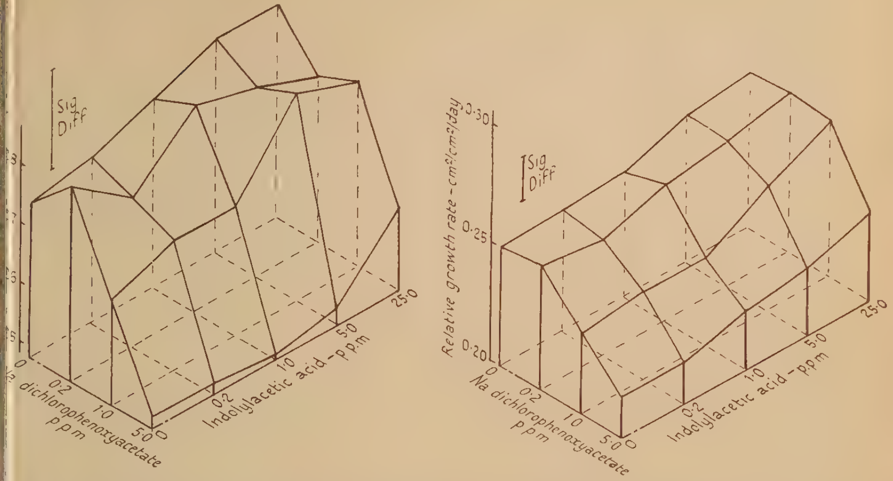
The morphological effects at the lower concentrations were similar to those in Expt. 1, but were more pronounced at the higher concentrations. At 5 and 25 p.p.m. of indolylacetic acid the daughter fronds remained attached to the parents by filaments of tissue, while a marked degree of 'humping' was caused by these two concentrations. At lower concentrations the colour of the fronds, compared to that of the controls, was darker, but at 25 p.p.m. the fronds became slightly chlorotic. This effect on the colour of the fronds was not masked by the highest concentration of the phenoxyacetate. In the absence of indolylacetic acid the two higher concentrations of sodium dichlorophenoxyacetate caused a progressive diminution in the area of individual fronds, while in its presence this effect was less.

At each level of indolylacetic acid, increases in the concentration of the phenoxyacetate above 0·2 p.p.m. caused progressive reductions in both area and weight (see Figs. 4 and 5). On an area basis the reductions were significant at 1 p.p.m. while, on a weight basis, significant decreases did not occur below a concentration of 5 p.p.m. The magnitude of the reductions did not vary to any appreciable extent between the levels of indolylacetic acid and there was no significant interaction between compounds.

At all levels of the dichloro compound the growth rate for area rose as the concentration of indolylacetic acid increased, the increases reaching significance at a concentration of 5 p.p.m. In this instance the increase was significantly greater at the highest levels of the phenoxyacetate. The changes in the relative growth rate for weight (Fig. 4) showed similar trends. Expt. 2 provides some evidence that the presence of sodium dichlorophenoxyacetate has a slight influence on the changes induced by indolylacetic acid. Since the growth rates are greater when most auxin is supplied and as the order of depression due to the phenoxyacetate is approximately the same for each level of indolylacetic acid, it follows that the percentage depression is less at the higher levels of the auxin. Conversely, relative to the controls, increasing the auxin supply causes more marked changes in the presence of the highest levels of sodium dichlorophenoxyacetate.

In order to examine in greater detail these interacting trends, the growth rates for changing area have been expressed on a somewhat different basis. To assess the effect of the auxin at each level of the phenoxyacetate, the relative growth rates at the four concentrations of indolylacetic acid (in each block) have been expressed as a percentage of the rate in the absence of the auxin. From Fig. 6 it is evident that, irrespective of the level of the phenoxyacetic acid, there is with an increase in the concentration of indolylacetic acid a rising trend. However, the increase in the presence of 1 p.p.m. or, to a lesser extent, of 5 p.p.m. sodium dichlorophenoxyacetate, is significantly greater than the corresponding increase in its absence.

If the data are treated in a similar fashion but relating the changes to those



FIGS. 4 and 5. Expt. 2. The effects of various combinations of sodium 2 : 4-dicholorophenoxyacetate and 3-indolylacetic acid on the relative growth rate of *Lemna minor*. Fig. 4 on weight, Fig. 5 on area basis.

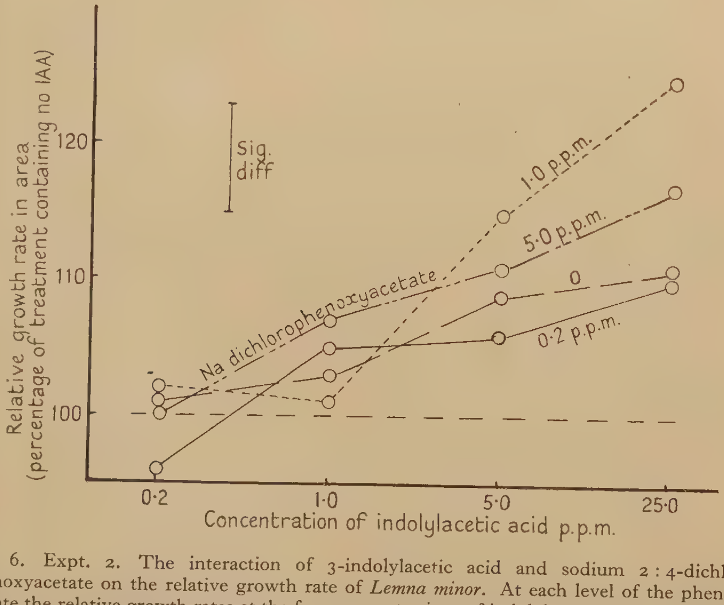


FIG. 6. Expt. 2. The interaction of 3-indolylacetic acid and sodium 2 : 4-dichlorophenoxyacetate on the relative growth rate of *Lemna minor*. At each level of the phenoxyacetate the relative growth rates at the four concentrations of indolylacetic acid are expressed as percentages of the rate in the absence of the auxin.

found in the absence of sodium dichlorophenoxyacetate, then it can be shown that the reduction due to the phenoxyacetate is significantly less at relatively high concentrations of indolylacetic acid.

Statistical analysis of the ratios of area to weight indicated that a highly significant variation could be attributed to concentration for both compounds ($P < 0{\cdot}001$), while the interaction was also significant. The overall effects are similar to those in the first experiment, that is, with increasing concentration the phenoxyacetic acid decreases the ratio and the auxin augments it (Fig. 7). At the three lower levels of the phenoxyacetate the increase caused by the auxin is significant at 5 p.p.m., but at the highest level of the synthetic growth regulator the corresponding significance is at 1 p.p.m. Thus once more an interdependence between the two compounds is apparent.

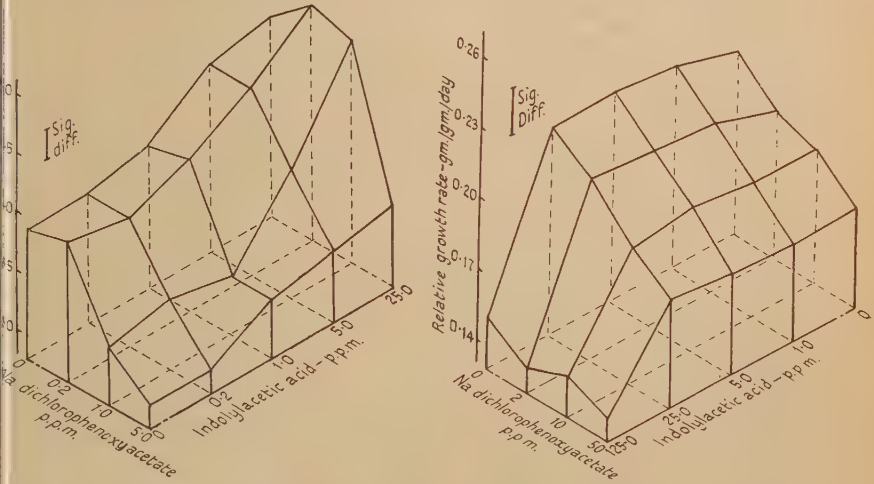
In both the previous experiments the levels of indolylacetic acid had not been sufficiently high to reduce the relative growth rate. Hence a further experiment was undertaken with still greater concentrations, i.e. a range of 0, 1, 5, 25, and 125 p.p.m. combined factorially with 0, 2, 10, and 50 p.p.m. of sodium dichlorophenoxyacetate. As it was found that the higher concentrations produced very marked epinasty, no measurements of area were made since the method of area measurement was not suitable for severely deformed fronds.

During the first 24 hours of the experiment a thermal relay switch controlling one of the thermostats failed during the night and caused the bath temperature to rise. The period could not have exceeded 8 hours, by which time the temperature for baths I and II had risen to 35° C. while that of bath III was 33° C. Owing to the arrangement of the circulation system of the three baths, the onset of this high temperature would not have occurred at the same time in all blocks.

The formative effects caused by the lower concentrations were similar to those in the previous experiments, but at all levels of phenoxyacetate the highest concentration of indolylacetic acid caused pronounced injury during the first 48 hours and by the end of this period the fronds had split into individual units and exhibited a general chlorosis. This markedly phytotoxic effect had not been anticipated so that the concentration was subsequently reduced to 100 p.p.m. Even after this reduction, growth was greatly inhibited, and so at the end of the sixth day the concentration was further reduced to 75 p.p.m. until the ninth and final day of the experiment.

From Fig. 8 it is evident that, with increasing concentration of the dichloro compound, growth is progressively depressed, the order of the depressions being least at the highest concentrations of indolylacetic acid. Over all levels of the phenoxyacetate an increase in the supply of auxin up to 25 p.p.m. induced a gain in the growth rate. The greatest increase occurred in the presence of 2 p.p.m. of the dichloro compound, and this result is in good agreement with the trend of Expt. 2, in which the maximum increase of the growth rate (based on area) occurred at the level of 1 p.p.m. However, when, in the present experiment, the concentration of indolylacetic acid was raised above 25 p.p.m., a very large depression of the growth rate (based on weight) resulted.

As in Expt. 2, in order to bring out the interacting trends, the growth rates for one compound at any one level of the other compound have been expressed as a percentage of the corresponding rate of the cultures containing none of the first compound. When the percentages are based on the growth rates of the cultures containing no sodium dichlorophenoxyacetate, the concentration effects for both compounds are highly significant ($P < 0{\cdot}001$), but the interaction is not. From Fig. 9 *a* it is seen that an increase in the concentration of the phenoxyacetate causes a maximum decrease, on the percentage scale, at low levels of the auxin and brings about a minimum percentage depression at the highest auxin concentration.



FIGS. 7 and 8. Expt. 2. The effects of various combinations of sodium 2 : 4-dichlorophenoxyacetate and 3-indolylacetic acid on (Fig. 7) the ratio of frond area to total plant weight and (Fig. 8) on the relative growth rate of *Lemna minor.*

When the growth rates are expressed as percentages of those containing no indolylacetic acid (see Fig. 9 *b*), the concentration effect for the auxin but not for the dichlorophenoxyacetate is significant, while the interaction between concentrations is also significant ($P < 0{\cdot}01$). In the absence of the phenoxyacetate, or at 2 p.p.m., increasing the concentration of indolylacetic acid up to 25 p.p.m. causes a significant percentage increase in the growth rates, while at 10 or 50 p.p.m. of the phenoxyacetate there is no such effect. When, however, the concentration of the auxin is increased beyond 25 p.p.m., a large decrease takes place, but this is minimal at the higher concentration of the phenoxyacetate, the difference in effect between 2 and 50 p.p.m. being significant.

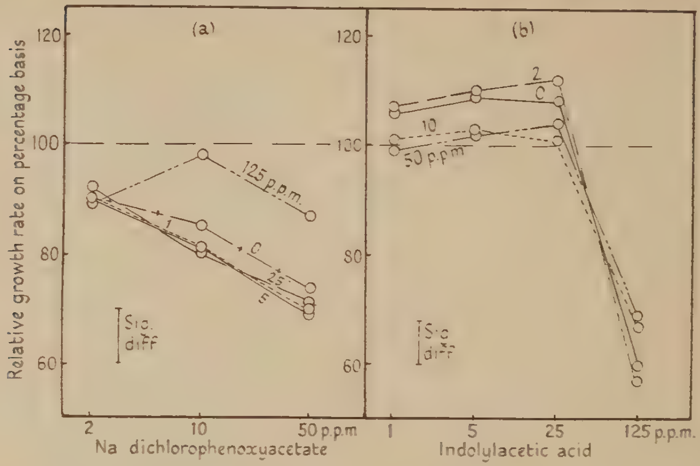


FIG. 9. Expt. 3. The interaction of 3-indolylacetic acid and sodium 2 : 4-dichlorophenoxyacetate on the relative growth rate of *Lemna minor*: (*a*) at each level of indolylacetic acid (0, 1, 5, 25, and 125 p.p.m.) the relative growth rates at the three levels of the phenoxyacetate are expressed as percentages of the rate in the absence of the phenoxyacetate; (*b*) at each level of sodium dichlorophenoxyacetate (0, 2, 10, 50 p.p.m.) the growth rates at the four levels of indolylacetic acid are expressed as percentages of the rate in the absence of the auxin.

The interrelated effects of 3-indolylacetic acid and sodium 2 : 4-dichlorophenoxyacetate on the growth of Helianthus annuus

For the sunflower, pilot experiments were first undertaken and these showed that single applications of up to 100 μg. of indolylacetic acid, applied to the first leaf, caused no measurable change in the production of dry matter. Applications of this order induced both epinastic responses and alterations in the rate of internode elongation, but they did not appear to influence the nature of the response arising from treatment with sodium dichlorophenoxyacetate. A further experiment, in which up to 100 μg. of indolylacetic acid were applied each second day, also indicated that the highest dose caused a significant reduction in the relative growth rate of both the young leaves and the second internode, but none caused a significant increase in any of the component parts.

In the main experiment 4 levels of indolylacetic acid (0, 6, 30, 150 μg. plant) were combined factorially with 3 levels of sodium dichlorophenoxyacetate (0, 20, 100 μg. plant). Each compound was applied in aqueous solution to the first leaves, the concentration of the phenoxyacetate being 0·25 per cent. and that of the auxin 0·3 per cent., while the amounts were varied by varying the volume applied. To increase the magnitude of the effects induced by the indolylacetic acid the applications were repeated at daily intervals.

Within a few days plants treated with the phenoxyacetate showed marked epinastic effects and a retardation of leaf expansion. Some 12 hours after the first application the leaves above the pair treated with indolylacetic acid assumed a vertical position, but during the following few days returned to a normal position which was not greatly altered by further applications. In addition, the length of the first internode (immediately below the treated pair of leaves) and the hypocotyl was reduced, but the length of the second internode was not altered by the end of 8 days when the pots were harvested and from the dry weights the relative growth rates of the various parts calculated.

In order to simplify the interpretation the results have been expressed relative to the rates of the untreated plants. It is seen from Fig. 10 *a* that a significant reduction in the relative growth rates of the treated (first) leaves is induced by the phenoxyacetate ($P < 0{\cdot}01$) and over all levels of indolylacetic acid there is a general trend for the magnitude to increase with the concentration. In addition, indolylacetic acid also reduced the growth rate, but in combination with the phenoxyacetate the apparent additional effects do not reach significance. The growth rate of the first internode (Fig. 10 *b*) is also significantly affected by both compounds, and from a comparison of Fig. 10 *a* and *b* it is of interest that the effects again appear to be additive, in that the greatest reduction occurs when the highest levels for both compounds are combined.

The changes in the hypocotyl are more complex (see Fig. 11 *b*): the influence of the phenoxyacetate in stimulating growth is highly significant ($P < 0{\cdot}001$), so is the interaction between compounds. With increasing indolylacetic acid there is a significant and progressive rise in the growth rate only at the intermediate level of 20 μg. of the phenoxyacetate; with 100 μg. the relative acceleration of the growth tends to fall at the higher levels of indolylacetic acid. Thus the two compounds have a complementary effect, first bringing about an increase and then a decrease when the combined dose is sufficiently high. From Fig. 11 *a* it is evident that the roots behave in much the same way as the first internode. Increasing the dose of either compound brings about a significant ($P < 0{\cdot}01$) reduction, a combination of the highest doses of both compounds causing the maximum depression.

When the parts above the point of application are considered, it is seen that the changes induced in the young leaves follow a similar pattern to those of both the treated leaves and the first internode (Fig. 12 *b*). Increasing doses of either growth regulator cause significant ($P < 0{\cdot}001$) depressions, the maximum reduction again resulting from a combination of the highest levels.

Like the responses of the hypocotyl, the reactions of the second internode are relatively complex (Fig. 12 *a*). At all levels of the auxin, as the dose of the phenoxyacetate is increased there is first an increase followed by a decrease in the growth rate. For plants receiving either no phenoxyacetate or 20 μg., increasing the dose of the auxin brings about a reduction; but at the highest level of the dichloro compound as the auxin level is raised there is a slight, though not significant, increase followed by a decrease.

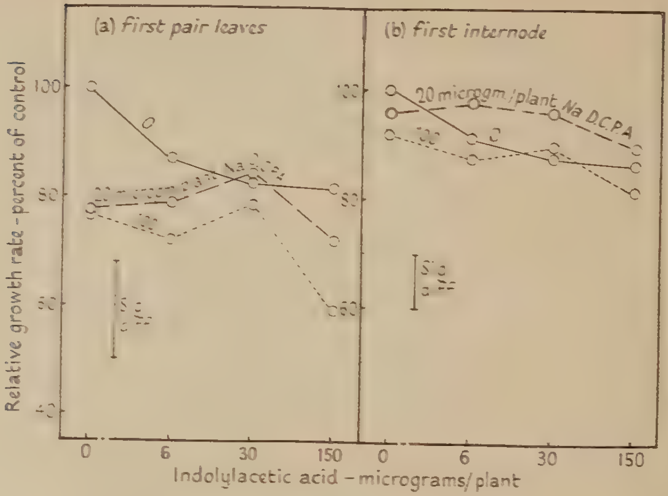


FIG. 10.

FIGS. 10–12. Expt. 6. The interaction of 3-indolylacetic acid and sodium 2 : 4-dichlorophenoxyacetate on *Helianthus annuus*. The effects of various levels of the phenoxyacetate (0, 20, 100 μg./plant) on the changes in the relative growth rate brought about by increasing doses of indolylacetic acid on Fig. 10 (*a*) the first leaves, Fig. 10 (*b*) the first internodes; Fig. 11 (*a*) the roots, Fig. 11 (*b*) the hypocotyls; Fig. 12 (*a*) the second internodes, Fig. 12 (*b*) the second leaves.

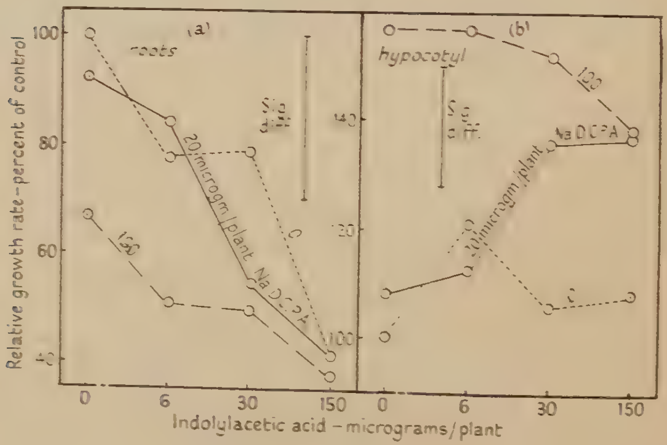


FIG. 11.

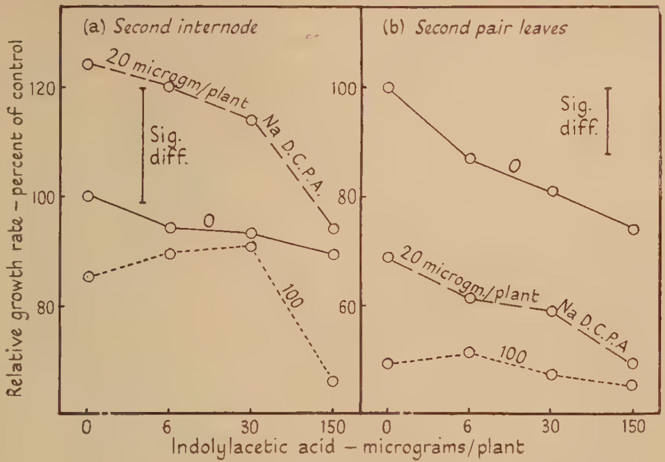


FIG. 12.

DISCUSSION

The present investigations have shown that between the selected species there are marked contrasts in the effects induced by the two growth regulators. It is also evident that the experimental conditions were widely divergent, but on the basis of the changes arising from (*a*) an increase in the supply of indolylacetic acid to both species, and (*b*) the introduction of dichlorophenoxyacetic acid, a comparison of general trends is still valid. However, in the case of *L. minor* the cultural technique did not involve aseptic conditions and it might be postulated that the increases in the growth rate brought about by indolylacetic acid were not direct effects but secondary effects associated with the presence of micro-organisms, largely bacteria. Such indirect effects seem unlikely. Firstly, any increase in bacteria would tend to compete for the nutrients and thereby reduce the growth rate of *L. minor*. Secondly, since the pH was adjusted initially to an optimal value, any shift resulting from the activities of micro-organisms would again tend to reduce rather than increase growth. Thirdly, although the possibility that the bacteria themselves produced growth stimulatory substances cannot be ruled out, the production of such a growth factor seems improbable.

When comparisons between species are made it is clear that the growth rate of *L. minor*, either on the criterion of weight or area, is accelerated by low doses of indolylacetic acid. On the other hand, in the sunflower, save for the hypocotyl, an increase in the supply depresses the growth rates of the component parts and as a net effect decreases the growth rate of the whole plant. It would be advanced that these specific differences are associated with differences in the available supply of auxins and the levels required for optimum growth. Since both the hypocotyl of the sunflower and *L. minor* grow faster when there are small increments in the supply of indolylacetic acid, it would suggest that the tissues contain suboptimal and limiting levels of the auxin. On the other hand, as the addition of indolylacetic acid causes a retardation of growth in the remaining parts of the sunflower, they seemingly contain at least an optimal level.

Further specific differences are indicated by the differential reactions to the two growth regulators. It has been demonstrated that most parts of the sunflower react in a similar manner to both compounds, but with *L. minor*, though the growth rate is increased by some levels of auxin, it is depressed by the phenoxyacetate. Moreover, it is unlikely that, if a wider range of doses of sodium dichlorophenoxyacetate had been employed, an increase in the growth rate would have been recorded, since in Expt. 1 the two lowest concentrations had no significant effects.

Before considering the interaction of the two compounds it will be recalled that in Expt. 6 the response of the hypocotyl in sunflower plants receiving only the phenoxyacetate was maximal at the highest concentration tested. However, other experiments have shown that at higher doses the growth rate again falls. Thus the response to increasing concentration is typical of the reactions of stem tissue to this and other growth regulators.

Although in the sunflower both compounds depress the growth rates of the treated leaves, the first internode and the roots, the effects of the combined doses are not wholly additive, that is to say, the inhibitory effect of the combined highest doses is not equal to the sum of the separate effects. It is true that the greatest depression occurs when the highest doses of both compounds are applied concurrently, but the percentage change in the growth rate caused by increasing the dose of either compound is greater if the other is absent. When the phenoxyacetate is either not applied or given in small amounts, increasing the amount of indolylacetic acid accelerates the growth rate of the hypocotyl. On the other hand, at the highest level of the dichloro compound, growth is depressed as more auxin is added. However, this complex response does not necessarily indicate that the two compounds act in a different manner. When the sum of the combined doses is small, the total amount of growth regulator reaching the hypocotyl might still result in a stimulation of growth. But as the amount of each component is increased, an acceleration in the growth rate will in turn be replaced by a retardation when a high and inhibitory level is reached.

The depressions in the growth rate of the young leaves due to the combined action of both compounds are very similar to those of the older leaves which received the droplet applications. Such a similarity does not necessarily imply that the effects are directly comparable. Goodall (1945) has shown for the tomato that the maximum growth of young leaves is dependent on the transport of carbohydrates from older leaves. Thus a reduction in the area of treated leaves and the consequent decrease in carbon fixation would reduce the carbohydrates available for transport to the young leaves.

When the reactions of the second internode are assessed, it appears that the two compounds must act in different ways. The curve of response with increasing phenoxyacetate has a typical peak and it can be inferred that this compound has a direct action. On the other hand, the reductions in growth due to indolylacetic acid can be ascribed to two possible causes: a direct effect of an inhibitory level, and an indirect effect due to the reduction in leaf area and the consequent smaller carbon fixation. In this connexion there is a further factor which needs taking into account, namely, differential transport from the treated leaves. Boysen Jensen (1936) was the first to point out that when indolylacetic acid is applied to stem segments transport is polar. In other experiments which have been concerned with the penetration of sodium 2 : 4-dichloro-5-iodo-phenoxyacetate (containing the radioactive isotope I^{131}) into sunflower leaves, it has been found that I^{131} first accumulates in the stem below the treated leaves but even within 6 hours activity can be detected in the upper part of the shoot, and within 48 hours the iodine is generally distributed in the shoot with appreciable accumulation in the youngest leaves. Although the movement of the iodine does not necessarily reflect the transport of the intact molecule, the data suggest that there is first a polar movement out of the leaves through the phloem and that subsequently there is upward movement of the phenoxyacetic acid or some breakdown product in the phloem or via the transpiration stream. It may well be that the pattern of the transport of the applied indolylacetic acid may be different, such that there is differential accumulation of two compounds in the second internode or younger leaves. However, as the young leaves above the point of application assumed a vertical position shortly after treatment with indolylacetic acid, it is more reasonable to suppose that the auxin or some accessory compound can be translocated rapidly upwards than to assume that the changes in the leaf position were secondary effects induced by some action of the auxin in a lower part of the stem. Nevertheless as the elongation of the first internode is depressed more than that of the second internode, it is probable that translocation downwards is greater.

Turning to *L. minor* it would seem that at low concentrations the effect of one compound is influenced by the simultaneous application of the other. The effects of indolylacetic acid on the response to the phenoxyacetate are like those observed for most parts of the sunflower—that is, the percentage depression caused by the phenoxyacetate is lessened in the presence of the auxin. On the other hand, the influence of the phenoxyacetate on the response of the auxin is not always similar; high concentrations diminish the effect of the auxin but low concentrations increase the response. Such an increase occurs when the criterion is either the relative growth rate or the ratio of area to weight.

Before seeking to interpret further the interacting trends, some discussion of the recent work of Hitchcock and Zimmerman (1952) is relevant. They too studied the reactions of the tomato to indolylacetic acid and dichlorophenoxyacetic acid, more particularly under the conditions when droplets were applied to the leaves, and chose as their criteria of response the direct measurement of stem curvature and the total height of the shoot, together with a scoring technique for assessing leaf modification and stem proliferation. The main conclusions reached were that dichlorophenoxyacetic and indolylacetic acid when applied alone produced the same qualitative and quantitative responses, save that the auxin did not bring about leaf modification. Dependent upon whether the auxin induced an accelerating or inhibitory effect, the reactions brought about by combinations of the two compounds were equivalent to those brought about by smaller or larger amounts of the phenoxyacetate. Their results provided clear evidence of a synergistic effect on leaf modification since a level of auxin, which by itself had no effect, when added to the dichloro compound appreciably reduced the changes in leaf shape. For the remaining observations, where indolylacetic acid produced responses, statistical evidence of the significance of the interactions between compounds was not provided and the non-orthogonal design of most of the experiments makes interpretation difficult. The changes in stem height caused by the two compounds appear to be additive, but the statement that growth and increased height can be used synonymously does not appear valid in view of the differential effects obtained in the present experiments for the growth of the hypocotyl and the different internodes. In fact, Hitchcock and Zimmerman themselves record that the degree of stem proliferation below and above the treated leaf is different and that the addition of the auxin to the phenoxyacetate brings about varying effects between the first and sixth internodes.

From the foregoing discussion it is clear that the concept of wholly additive effects of the two compounds fails to explain many of the observed results. Indeed, it would appear that the compounds can mutually interfere in their actions, and the conclusion must be reached that more than one mechanism is involved. Firstly, it may be postulated that the two compounds act on a common system and that they compete with one another. At least with *L. minor*, one common system may relate to the uptake mechanism. Thus if the presence of sodium dichlorophenoxyacetate depresses the amount of indolylacetic acid absorbed by the fronds, then the increase in growth resulting from the addition of the auxin will be minimized or nullified by the addition of the dichloro compound to the culture. For stem tissue and carrot disks, current work in this laboratory shortly to be published (Reinhold) is in agreement with the postulate that the dichlorophenoxyacetic acid depresses the uptake of indolylacetic acid. Whether the reverse trend is true is not yet known.

In the sunflower experiments, since the growth regulators were applied in separate solutions it cannot be advanced that there is a mutual interference on the basis of penetration into leaves. Nevertheless, many of the results are compatible with the concept that both compounds act on the same system and that they compete at the site or sites of reaction. In this connexion unpublished research on the relationship between the phytotoxicity and chemical and physical characteristics of substituted phenoxyacetic acids indicates that there is a broad and inverse relationship between toxicity to *L. minor* and solubility in water. Thus physical adsorption may well be an important factor and this suggestion is in line with the concepts of Booij and Veldstra (1949).

A further hypothesis which needs discussion is that each compound brings about the inactivation of the other. In 1949 Goldacre claimed that *in vitro* the enzymatic destruction of indolylacetic acid was accelerated in the presence of 2 : 4-dichlorophenoxyacetic acid. More recently Galston, Weintraub, and Goldacre[1] (1953) have found that the acceleration was not due to the dichlorophenoxyacetic acid itself but to the presence, as an impurity, of 2 : 4-dichlorophenol. If, *in vivo*, there is such a conversion, even in part, to the parent phenol, then it might be expected that there will be some linkage between the relative toxicities of substituted phenoxyacetic acids and the comparable phenols. Parallel studies by one of us (G. E. B.) working with Serafin Novoa-Quintas on phenoxyacetic acids and with M. H. Parke[2] on substituted phenols do not suggest that for *L. minor* there is a clear-cut linkage, when the activity is measured in terms of the chlorotic effects produced under standard conditions (see Blackman and Robertson-Cuninghame, 1953, for further details). Some preliminary supporting evidence is given in Table I, where it can be observed that the ratio of toxicity between pairs of compounds varies more than a hundredfold.

TABLE I

The relative toxicities of substituted phenoxyacetic acids and the parent phenols to Lemna minor

	Molar concentrations required to produce standard degree of chlorosis		
Substitution	Phenoxyacetic acid	Phenol	Ratio acid/phenol
—	$27·4 \times 10^{-4}$	$1·6 \times 10^{-2}$	0·17
2-methyl-	$13·2 \times 10^{-4}$	$6·9 \times 10^{-3}$	0·19
2 : 4 dimethyl-	$8·4 \times 10^{-4}$	$2·4 \times 10^{-3}$	0·35
2 : 4 dichloro-	$3·3 \times 10^{-4}$	$3·5 \times 10^{-4}$	0·95
2 : 4 : 6 trichloro-	$1·25 \times 10^{-4}$	$3·0 \times 10^{-5}$	4·27
2 : 4 : 5 trichloro-	$1·63 \times 10^{-4}$	$8·4 \times 10^{-5}$	19·40
2 : 4 dichloro-6-methyl-	$1·29 \times 10^{-4}$	$3·7 \times 10^{-4}$	0·35
2 : 6 dimethyl-4-chloro-	$1·25 \times 10^{-4}$	$4·2 \times 10^{-4}$	0·30

The results of Table I do not, however, rule out the possibility that the level of indolylacetic acid is not depressed through some action of dichlorophenoxyacetic acid. Such an assumption could account for several of the observed facts: firstly, if the supply of the auxin is at a normal or suboptimal level, then the addition of the phenoxyacetic acid would reduce the level and decrease growth. Secondly, if the level is supra-optimal and inhibiting, then incremental additions of the dichlorophenoxyacetic acid would accelerate growth up to a certain maximum. On the basis of the first proposition, the

[1] Private communication prior to publication.

[2] Thesis for Degree of Bachelor of Science (1953).

trends for *L. minor* are in line with the hypothesis, while the changes observed in the second internode of *H. annuus* are in agreement with the second.

With other parts of the sunflower, such as the roots or first internode, it has already been put forward that as indolylacetic acid depresses growth, the supply of indolylacetic acid is optimal or supra-optimal, and yet contrary to the hypothesis even the lowest doses of dichlorophenoxyacetic acid depress growth. There is, however, the complication that if the first leaves contain an optimal auxin level then dichlorophenoxyacetic acid will on the hypothesis reduce leaf growth, will thereby diminish carbon fixation and in consequence the supplies of carbohydrate available for the growth of the stem and roots.

Even, however, when a reduction of carbohydrate supplies is taken into account, the changes in the hypocotyl cannot be readily fitted into the framework of the hypothesis. It will be recalled that although additional indolylacetic acid brought about gains in weight, i.e. the initial level was sub-optimal, yet at low levels of the dichlorophenoxyacetic acid growth was increased.

Although the results of this investigation do not indicate that the primary action of dichlorophenoxyacetic acid is to inactivate indolylacetic acid, yet it is still possible that such an effect is but one of the many phases of the action of this compound. Indeed, from the foregoing discussion it would appear that under some conditions the two compounds may supplement each other while under others they may be directly competitive. Moreover, since the recent findings of Bennet-Clark *et al.* (1952), Jones *et al.* (1952), and Luckwill (1952) have demonstrated that besides indolylacetic acid there are present in plant tissues both other growth regulators and growth inhibitors, the dichlorophenoxyacetic acid may well react with any of these compounds and so upset the balance of factors controlling growth.

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Effects of 3-Indolylacetic Acid and 3-Indolylacetonitrile on the Growth of Excised Tomato Roots

H. E. STREET, SHEILA M. McGREGOR
AND I. M. SUSSEX
Department of Botany, Manchester University

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SUMMARY

1. The inhibition by IAA (3-indolylacetic acid) and by IAN (3-indolylacetonitrile) of the growth of excised tomato roots cultured for 7 days at 27° C. in a modified White's medium is described. 5×10^{-9} g. ml. IAA or 5×10^{-6} g./ml. IAN cause approx. 50 per cent. inhibition of the linear growth of the main axis. With IAA decrease in number of laterals closely parallels the decrease in linear growth of the main axis; with IAN reduction in linear growth of the main axis occurs at concentrations above 10^{-8} whereas number of laterals does not decrease until the concentration exceeds 10^{-6}.

2. Study of the course of cell elongation in the exodermal cells showed that in the standard medium and in media containing 5×10^{-9} IAA or 5×10^{-6} IAN the process takes about 7 hours; the final cell lengths in IAA and IAN media are lower than in standard medium owing to a slower rate of elongation. The decrease in linear growth of the main axis in presence of IAA could be accounted for by the decrease in cell length; this was not the case with IAN. The implications of this are considered.

3. Determinations of the distance (mm.) between, and of the number of exodermal cells separating, the adjacent laterals in one orthostichy showed that IAN enhances the frequency of laterals whereas this is either unaffected or decreased by IAA. The enhancement of lateral frequency in IAN arises from shortening of the cells of the main axis and decrease in the number of cells separating adjacent laterals.

4. The results are considered to support the view that IAN has effects on root growth different from those of IAA. Study of the degree of inhibition of main axis growth and of alterations in lateral frequency resulting from treatment with mixtures of IAA and IAN provided data which could also be most easily explained on this hypothesis.

INTRODUCTION

PREVIOUS studies in our laboratory (Street and Roberts, 1952) have provided evidence that the main axis meristem of the excised tomato root checks the growth of laterals, particularly of those nearest to the root apex. At the same time it was shown that the duration of activity of the main axis meristem is prolonged by repeated excision of the main root-tip. This indicated that the activity of the main axis meristem is in turn influenced by the older part of the main axis with its associated laterals. The survival of the main axis meristem when repeatedly subcultured has also been shown to be markedly affected by the sucrose concentration of the medium (Street, McGonagle,

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and Roberts, 1953), and by illumination of the roots (Street, 1953). These observations led us to adopt the working hypothesis that the duration and level of activity of the meristems of excised roots is controlled by auxins and anti-auxins and that excision and cultural conditions influence meristematic activity by affecting the hormonal balance within the root. Recent work by Bennet-Clark and Kefford (1953) indicates the occurrence in a number of roots of 3-indolylacetic acid (IAA), accelerator-α, inhibitor-β, and possibly also of 3-indolylacetonitrile (IAN). Progress in our knowledge of the hormonal control of root growth now clearly involves defining the effects not only of absolute concentrations of these substances but of the balance between them.

Examination of the hormonal control of meristematic activity in excised roots must, therefore, involve both study of the effects of natural hormones, separately and in combinations, on root growth and interpretation of the effects of these and other cultural treatments in terms of their influence on the hormone content of the roots. As a contribution to such an examination the present paper describes some effects on excised root growth of external applications of IAA and IAN, the only two natural root hormones at present available in pure form.

EXPERIMENTAL

General experimental procedure. The general culture technique has been previously described (Dormer and Street, 1949; Street and Lowe, 1950). The standard medium was a modified White's culture solution (Street and McGregor, 1952), sterilized by autoclaving at 15-lb. pressure for 5 minutes. IAA and IAN were incorporated in this medium before autoclaving. Concentrations of IAA and IAN are expressed throughout as g. per ml. of culture medium. The culture flasks, containing 50 ml. of medium, were each inoculated with a 10 mm. root-tip excised from 7-day sector cultures of a clone of tomato roots ('Best of All'). Unless otherwise stated the cultures were incubated for 7 days at $27\pm0{\cdot}5^\circ$ C. At the end of this period roots were harvested and measured. Three features of the roots were recorded: increase in length of main axis (final length of main axis minus initial length of inoculum), number of laterals, and total length of laterals. In addition, in some experiments distance from the root apex to the first visible lateral was also recorded.

All culture media were of initial pH 4·4–4·6. Standard medium which had supported the growth of a root for 7 days had a pH of 5·2–5·6. Media containing IAA and IAN at inhibitory concentrations showed a less marked pH drift.

The IAN in standard medium (at 10^{-5} g./ml.) was examined for its stability to autoclaving (15 lb./5 min.) by means of the *Avena* coleoptile straight growth test (Bentley, 1950). The growth-promoting activity of the ether-extractable fraction of the medium was determined using medium before and after autoclaving. Autoclaving caused no change in activity. Tests were also carried out to see if any acid growth-promoting substance was produced from the nitrile during autoclaving. Acid and neutral extracts of the medium were prepared by the procedure of Bentley and Housley (1952). Full recovery of IAN was obtained from autoclaved medium rendered neutral by addition of 1 per cent. sodium bicarbonate. The subsequently prepared acid fraction had some growth-promoting activity, but this was not increased by autoclaving of the medium. The results indicate that IAN suffered no destruction during autoclaving.

The inhibition of root growth by IAA and IAN. The effects of IAA and IAN on root growth are shown in Figs. 1, 2, and 3. The results for increase in main axis length (Fig. 1) are similar to those reported earlier by Bentley and Bickle (1952). These workers studied the elongation of radicles (initial length 4 or 5 mm.) of *Lepidium sativum* during a 24-hour period by a modified form of the Moewus (1949) cress test. With excised tomato roots 50 per cent. inhibition of increase in length occurs at approx. 5×10^{-8} g. ml. IAA and at approx. 5×10^{-6} g. ml. IAN.

When (Fig. 3) increase in main axis length and lateral number (both as percentage of control) are plotted together it is seen that with IAA there is a fairly close parallel between the values. However, with IAN reduction of main axis length occurred at concentrations above 10^{-8} g. ml., whereas lateral number did not decrease until the concentration exceeded 10^{-6} g. ml. This differential effect of IAN on increase in main axis length and on lateral number is also apparent when the curves for increase in main axis length (Fig. 1) and for total length of laterals (Fig. 2) are compared. This increase in lateral frequency caused by IAN over the range 10^{-8}-10^{-6} g. ml. has been confirmed on a number of occasions and is considered in greater detail on p. 209. No similar effect is observed with IAA.

The effect of IAA and IAN on cell elongation. The course of cell elongation in the exodermis in the presence of IAA and IAN has been studied using a method previously described (Street and McGregor, 1952). Cell elongation is followed by determining cell lengths of exodermal cells at selected distances from the promeristem. The distances cover and extend beyond the region of cell elongation. At each distance 10 cells are measured to give a mean value, and for each treatment 5 roots are examined. In the present work each root was measured through the glass of the culture flask on the 4th day of incubation and again 24 hours later. The roots were then fixed in 70 per cent. alcohol, cleared with lactophenol, and mounted for measurement of the cell lengths. If it is assumed that active cell division is restricted to the region of the promeristem, then the values for increase in main axis length during the last 24 hours of incubation can be used to convert distances from the promeristem into hours separating the origin of the cells in the promeristem and the measurement of their cell lengths. Fig. 4 shows, using such a time scale, the course of exodermal cell elongation in roots grown in standard medium (control treatment), in medium containing 5×10^{-8} g. ml. IAA, and in medium containing 5×10^{-6} g. ml. IAN. Assessed over the total growth period of 5 days these concentrations of IAA and IAN caused, respectively, 45·9 and 52·3 per cent. inhibition of growth in length of the root. The mean

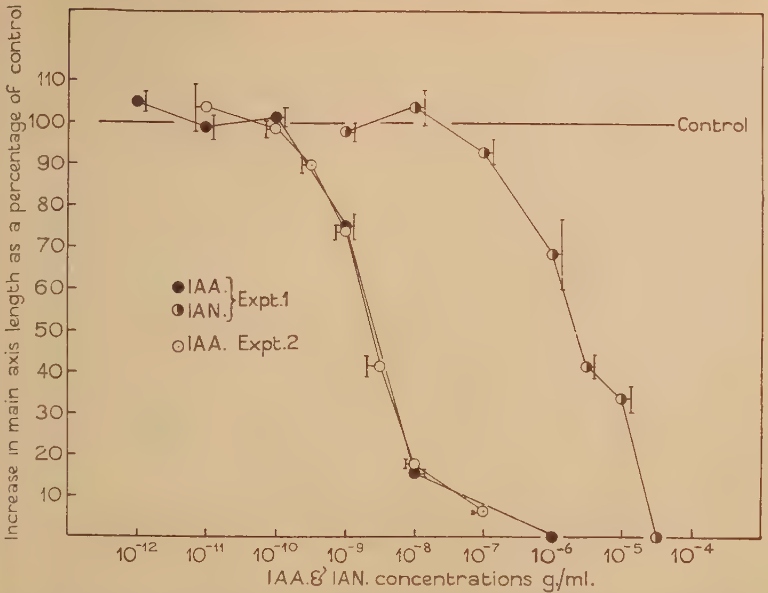

FIG. 1. Increase in length of main axis. Experiments 1 and 2 carried out on separate occasions. Replicate number for Expt. 1 is 19–20; for Expt. 2 is 14–15. Roots grown for 7 days at 27° C. The vertical black lines represent twice the standard errors of the mean values.

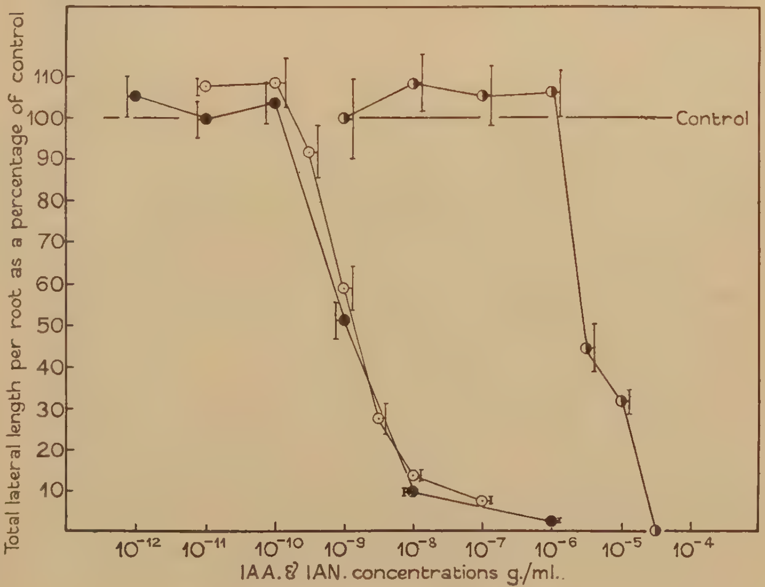

FIG. 2. Total length of laterals. (Key as Fig. 1.)

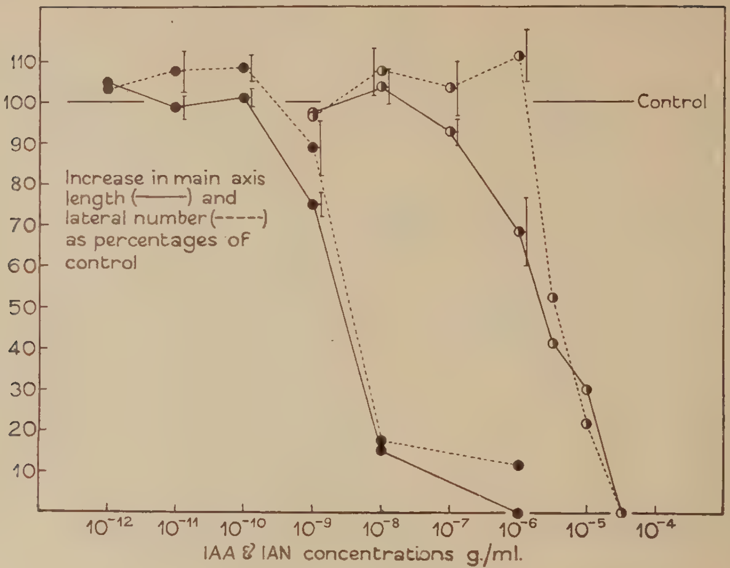

FIG. 3. Increase in length of main axis and lateral number. (Key as Fig. 1.)

increases in length of the main axis during the 24 hours immediately preceding harvesting were for standard medium 10·2 mm., for IAA medium 5·0 mm., and for IAN medium 5·1 mm. Fig. 4 shows that in each case the process of cell elongation occupied about 7 hours and that the lower values for cell length in IAA and IAN arise from a slower rate of cell elongation. If the inhibition of growth in length by these hormones is due to decrease in final cell lengths, then growth and cell length should correspond when expressed as a percentage of the control. If inhibition of growth also involves an effect on the number of cell divisions occurring in the meristem per unit time, then the decrease in cell length will be proportionately less than the decrease in linear growth. The lower rate of linear growth in the medium containing 5×10^{-9} g./ml. IAA (49 per cent. of the control) during the 24 hours prior to harvesting corresponds closely with the lower values for cell length (55 per cent. of control), indicating that the inhibition of growth is due to the decrease in cell length caused by the hormone. Reduction in cell-division rate appears, however, to be an important factor in the reduction of linear growth rate occurring in the medium containing 5×10^{-6} g./ml. IAN (50 per cent. of control), since in this treatment cell length is only reduced to 78·7 per cent. of the control value. The results presented in Table III (last two columns) also clearly point to the same conclusion. Since the process of main axis growth involves cell division and cell elongation and since the process of cell elongation is of short duration it can be concluded that, at concentrations causing up to 50 per cent. inhibition, IAA acts by causing a decrease in cell length and IAN acts by causing a less marked decrease in cell length accompanied by a decrease in division rate. Present evidence for such a differential effect of these two hormones on cell elongation and cell division is, however, indirect, and it is a necessary part of future work to compare their effects on mitosis frequency and duration. At high concentrations both hormones inhibit both cell elongation and cell division and when inhibition is complete all the cells of the meristem are seen to be vacuolated.

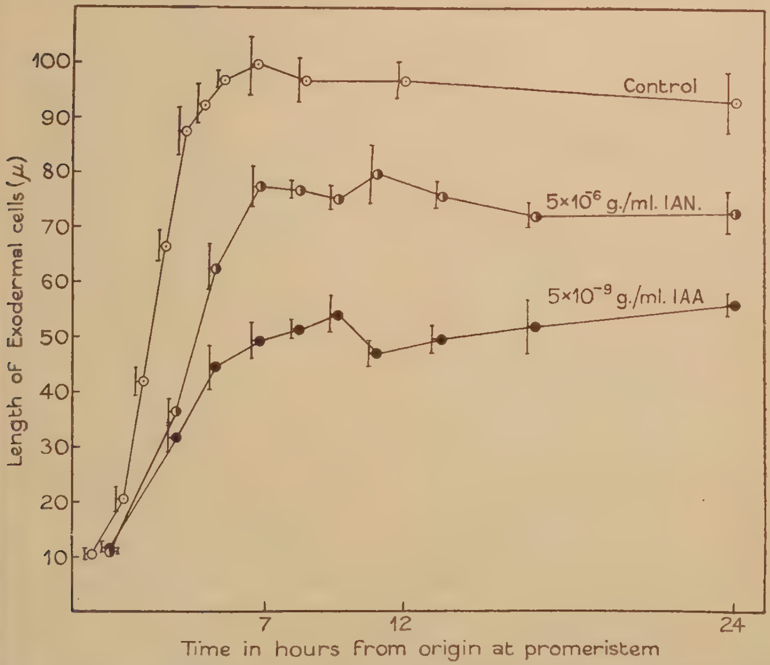

FIG. 4. The course of cell elongation in the exodermis during the 5th day of incubation of excised tomato roots growing in standard medium and in this medium modified by the addition of 5×10^{-9} g./ml. IAA or 5×10^{6} g./ml. IAN. Standard errors indicated as in Fig. 1. Replicate number is 5.

The effects of IAA and IAN on lateral root formation. As indicated on p. 206, there occurred in IAN media at 10^{-8} g./ml.-10^{-6} g./ml. a reduction of main axis length without a proportionate decrease in lateral number (Fig. 3). In the present section experiments are described showing that this is not primarily due to an increase in the proportion of the main axis carrying emerged laterals, but that the laterals arise closer together in roots grown in IAN media than in standard or IAA media.

Excised tomato roots grown in standard medium or in IAA and IAN media causing only partial inhibition of growth have their laterals arranged in two regular lines separated by 180° (arising opposite the protoxylem groups of the diarch stele). The spacing of laterals has, therefore, been expressed either as:

$$\text{(i)} \qquad 2 \times \frac{\text{Length of main axis bearing laterals (mm.)}}{\text{No. of laterals}} \qquad \text{(Table I).}$$

$$\text{(ii)} \qquad 2 \times \frac{\text{Length of sector of main axis (mm.)}}{\text{No. of laterals on the sector}} \qquad \text{(Table II).}$$

These values are referred to as $A\ L$ values. They are a measure of the distance apart (mm.) of the laterals of an orthostichy. Low $A\ L$ values imply a high lateral frequency or density. The sectors referred to in (ii) above were portions of the main axis either (*a*) of 10-mm. length and chosen so that the youngest lateral included was at least 2 mm. long (Expt. No. F. 5), or (*b*) of 20-mm. length and chosen so that the proximal portion just included the youngest lateral of 3-mm. length (Expt. No. F. 6 and F. 7). The roots examined were grown in standard medium (control treatment) and in media containing IAA and IAN at concentrations causing various and similar degrees of inhibition of main axis growth; the concentrations were chosen by reference to the inhibition curves (Fig. 1).

IAA concentrations 10^{-10} and 10^{-9} g. ml. did not give significantly different $A\ L$ values from the control, but at 5×10^{-9} g. ml. the values were significantly increased (Tables I and II). With higher concentrations of IAA lateral number was further depressed and roots either developed a few widely separated laterals or none at all. The marked decrease in cell length resulting from IAA treatment would be expected to cause crowding of laterals along the main axis. The present results therefore indicate that IAA over the whole range of inhibition is depressing lateral development and that this effect increases in intensity with increase in IAA concentration.

IAN concentrations 10^{-7}, 10^{-6}, and 5×10^{-6} g. ml. gave $A\ L$ values *below* those of the controls. At 10^{-5} g. ml. or higher concentrations the result varied according to whether the $A\ L$ values were determined by method (i) or method (ii) above. $A\ L$ values greater than control were obtained by method (i) (Table I); but less than control by method (ii) (Table II). IAN causes the laterals to arise closer together (gives low $A\ L$ values), but at 10^{-5} g. ml. or higher concentrations the distribution of laterals is uneven. They are crowded together at certain points but are absent on adjacent portions of the main axis. The proportion of main axis devoid of laterals increases markedly at high IAN concentrations. $A\ L$ values calculated by method (ii) and using only sectors bearing a complement of laterals, therefore reflect the crowding of laterals at points where they arise, whereas values calculated by method (i) represent the mean distance between laterals over the main axis as a whole including the regions devoid of laterals. Microscopical examination of roots at 10^{-5} g. ml. IAN showed that, in the regions devoid of laterals, the pericycle had undergone divisions all along the axis instead of forming individual lateral root initials. A similar generalized cell division in the pericycle had also occurred at some points between lateral initials. The decrease in cell division rate in the main axis meristem caused by IAN (p. 209), seems to be correlated with stimulation of cell division in the pericycle.

TABLE I

Effects of IAA and IAN on lateral frequency (i)

$$A/L \text{ values} = 2 \times \frac{\text{Length of main axis bearing laterals (mm.)}}{\text{No. of laterals}}$$

Expt. No.	Treatments. IAA or IAN concentration (g./ml.)	Mean A/L values and standard errors	Replicates
F. 1	Control	5·04 ± 1·90	14
	5×10^{-6} IAN	3·06 ± 0·91	23
F. 2	Control	3·64 ± 0·48	8
	10^{-6} IAN	2·96 ± 0·52	9
	10^{-9} IAA	3·52 ± 0·23	9
F. 3	Control	3·34 ± 0·10	28
	10^{-7} IAN	2·52 ± 0·12	30
	10^{-6} IAN	2·26 ± 0·06	30
	10^{-10} IAA	3·64 ± 0·14	30
	10^{-9} IAA	3·59 ± 0·16	30
F. 4	Control	3·86 ± 0·12	18
	5×10^{-6} IAN	2·72 ± 0·20	18
	10^{-5} IAN	4·32 ± 0·61	16
	10^{-9} IAA	3·52 ± 0·21	17
	5×10^{-9} IAA	4·54 ± 0·20	18

TABLE II

Effects of IAA and IAN on lateral frequency (ii)

$$A/L \text{ values} = 2 \times \frac{\text{Length of sector of main axis (mm.)}}{\text{No. of laterals on the sector}}$$

Expt. No.	Treatment. IAA or IAN concentration (g./ml.)	Mean A/L values	Replicates
F. 5	Control	3·34 ± 0·24	10
	5×10^{-6} IAN	1·72 ± 0·10	10
	10^{-5} IAN*	1·30 ± 0·34	8
	10^{-9} IAA	3·70 ± 0·14	10
	5×10^{-9} IAA	4·10 ± 0·22	10
F. 6	Control	2·96 ± 0·08	18
	5×10^{-6} IAN	2·28 ± 0·08	17
F. 7	Control	3·20 ± 0·10	19
	5×10^{-6} IAN	2·36 ± 0·08	19

* Two sectors omitted from the calculation as they did not bear a complement of laterals.

The decrease in *A L* values (i.e. increase in number of laterals per unit length of main axis) appeared to be greater than would be expected to result from the decrease in cell length caused by the IAN concentrations employed. This suggested that lateral initials in the IAN-treated roots were separated by a smaller number of cells than in the control or IAA-treated roots. Counts were made of the number of exodermal cells between adjacent laterals on one side of the root. The cells were counted along a single file of average length cells (longitudinal files of exodermal cells do occur which are composed of characteristically shorter or longer cells than the average). The mean value obtained from each root was based on ten such counts. Each treatment mean was derived from the values for ten roots. The results are shown in Table III. The number of exodermal cells between adjacent laterals is reduced by IAN and enhanced by IAA treatment. The contrast in the effects of IAN and IAA is seen particularly at 10^{-5} and 5×10^{-9} g. ml. respectively: concentrations which in this experiment caused 51 per cent. inhibition of main axis length.

TABLE III

Effect of IAA and IAN on distance apart and number of exodermal cells between adjacent laterals of one orthostichy (*Expt. No. F. 8*)

Treatment. IAA or IAN concentration (g. ml.)	Replicates	Distance between laterals (mm.)	No. of exodermal cells between laterals	Calculated cell length (μ)	Cell length as percentage of control	Increase in main axis length as percentage of control
Control	10	3·2±0·17	39·5±3·5	81·3	—	—
5×10^{-6} IAN	10	2·0±0·12	25·8±1·1	66·5	81·7	65·1
10^{-5} IAN	8	1·7±0·09	25·3±3·7	65·4	80·4	49·3
10^{-9} IAA	10	2·7±0·26	38·5±2·3	70·8	85·3	85·3
5×10^{-9} IAA	10	3·7±0·31	82·2±5·9	45·0	55·3	49·1

Over the appropriate concentration ranges IAA causes a decrease and IAN an increase in lateral frequency. At low concentrations of IAA the shortening of the cells compensates for the increase in the number of cells separating adjacent laterals and the *A L* values do not differ significantly from the control values. At higher concentrations the suppression of lateral development is sufficient to cause the laterals to be more widely spaced. At low concentrations of IAN laterals are more closely spaced than in the controls due to shortening of the cells combined with decrease in the number of cells separating adjacent laterals. This enhanced lateral density is, therefore, in part due to the promotion of meristematic activity in the pericycle. At higher concentrations the normal development of lateral initials is replaced by generalized meristematic activity in the pericycle.

Interactions between IAA and IAN in their effects on root growth. The results reported above suggest that the nitrile and the acid exert separate effects on the growth of excised roots. Simultaneous application of the two substances would, on this hypothesis, be expected to reveal interactions between them in their effects on root growth. Experiments were therefore undertaken in which IAN, IAA, and mixtures of the two were incorporated into the culture media. Since both substances at appropriate concentrations inhibit growth in length of the main axis, these experiments were designed to include IAA and IAN treatments of equal activity as judged by this criterion and to compare the effects of the two applied simultaneously at such concentrations with the effects of the individual substances at double these concentrations. The results are shown in Table IV. The inhibitory effect on increase in main axis length of mixtures of IAA and IAN is clearly greater than when these substances are applied singly. When concentrations of IAA and IAN causing individually a similar degree of inhibition are applied simultaneously, the inhibition caused by the mixture is greater than that of either constituent used at double its concentration in the mixture. This result points to differences in the action of these two substances on the separate components of the total elongation process. The lateral number of roots grown in the IAA–IAN mixtures is equal or greater than the number recorded

TABLE IV

Interactions between IAA and IAN in their effects on the growth of excised tomato roots. In each experiment 10 replicates per treatment

Expt. No.	Treatment. IAA and IAN concentrations (g./ml.)	Increase in main axis length (mm.)	Number of laterals per root	Length of laterals per root (mm.)
S. 1	5×10^{-6} IAN	30·8	13·3	54·0
	5×10^{-9} IAA	27·6	7·0	22·0
	5×10^{-6} IAN + 5×10^{-9} IAA	9·9	8·0	15·4
	2×10^{-5} IAN	26·7	0·1	1·0
	10^{-8} IAA	15·7	5·2	11·6
S. 2	10^{-6} IAN	52·9	29·6	164·0
	10^{-9} IAA	56·9	27·8	151·0
	10^{-6} IAN + 10^{-9} IAA	34·9	27·1	91·0
	2×10^{-6} IAN	49·3	30·3	139·0
	2×10^{-9} IAA	49·9	20·9	68·0
	5×10^{-6} IAN	37·3	16·4	82·0
	5×10^{-9} IAA	29·7	8·2	28·0
	5×10^{-6} IAN + 5×10^{-9} IAA	8·6	7·8	17·0
	10^{-5} IAN	24·4	6·1	36·0
	10^{-8} IAA	14·6	6·8	13·0
S. 3	2×10^{-6} IAN	58·0	34·1	170·0
	10^{-9} IAA	59·5	35·1	185·0
	2×10^{-6} IAN + 10^{-9} IAA	36·3	30·4	116·0
	4×10^{-6} IAN	45·0	32·6	140·0
	2×10^{-9} IAA	52·6	27·0	116·0
	5×10^{-6} IAN	45·3	30·8	131·0
	2×10^{-9} IAA	52·6	27·0	117·0
	5×10^{-6} IAN + 2×10^{-9} IAA	21·9	19·4	66·0
	10^{-5} IAN	34·0	12·5	53·0
	4×10^{-9} IAA	41·8	20·6	68·0

for the double IAA concentrations despite the more marked reduction in main axis length occurring in the presence of the mixture. The promotion of lateral development by IAN is, therefore, not prevented by the IAA addition but when combined with the marked shortening of cells probably associated with the IAA treatment gives roots with an exceptionally high lateral frequency. These results supported the hypothesis that IAN and IAA have different effects on excised root growth.

The sample of synthetic 3-indolylacetonitrile used in the present work was kindly made available to us by Dr. G. F. Smith, at the time a member of the Department of Chemistry of this University. We also wish to express our thanks to Dr. J. A. Bentley and her colleagues for assistance in the *Avena* straight growth tests and to Miss D. M. Davies for technical assistance and for preparing the text-figures.

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The Absorption of Potassium Ions by Plasmolysed Cells

J. F. SUTCLIFFE

Department of Botany, King's College, London

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SUMMARY

The absorption of potassium ions by cells of red beetroot tissue plasmolysed in various media has been examined and compared with that of unplasmolysed cells under similar experimental conditions.

It is established that although the plasmolysing agents in themselves tend to promote the absorption of ions at the concentrations employed, the effect of plasmolysis is to inhibit the rate of potassium uptake. Evidence is provided that this is due to an increase in the rate of leakage of ions from plasmolysed cells, and to a reduction of gross uptake.

These results are discussed in terms of the structural and physiological changes which are associated with plasmolysis. It is concluded that alterations in the surface area, and thickness or density of the protoplasts, modifications of the vacuolar concentration of ions, and respiratory influences are all involved. Another factor, the nature of which has not been elucidated, also appears to be involved.

INTRODUCTION

It is well known that when plant cells are plasmolysed in a hypertonic solution of a suitable electrolyte, recovery gradually occurs through the absorption of ions. The rate of deplasmolysis may therefore serve to indicate the permeability of protoplasts to various substances, and it appears to have been first used for this purpose by de Vries (1871). Since that time several attempts have been made to develop the method quantitatively for the determination of ion uptake (Lepeschkin, 1908; Fitting, 1917; Höfler, 1918), and it remains a popular exercise for students in the laboratory. It is generally assumed that data so obtained indicate the absorptive capacity of cells in the unplasmolysed condition, but there is no direct experimental evidence to support this contention (Stiles, 1950). The present investigation was undertaken to examine the situation by comparing directly the uptake of potassium ions by normal and plasmolysed cells under comparable experimental conditions.

At the outset it is evident that plasmolysis causes a number of structural and physiological changes which may be expected to influence the course of ion absorption. There are, for example, inevitably changes in the thickness or density and surface area of the protoplasmic layer across which the movement of ions occurs. There is probably an increase in either thickness or density, or both, but the alterations in relation to surface area are possibly

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more complex. Myers (1951) has shown that the permeability of a normal plant protoplast to water is only about 5 per cent. of its permeability in the plasmolysed state, and this has been attributed to an increase in the effective surface area of the protoplast when contact with the cell wall is lost. Apart from this effect, the surface area of a plasmolysed protoplast clearly depends upon the degree of plasmolysis, in that it is reduced when the cells are more strongly plasmolysed.

The relationship between the surface area of a plant cell and its permeability to ions has not yet been completely elucidated. Jacques (1939) has shown that it is not an important factor controlling the rate of ion uptake by *Halicystis* coenocytes, and we have observed (Sutcliffe, 1952*b*) that the rate of accumulation of potassium ions by extending maize-root segments remains almost constant during the growth phase (cf. also Brown and Cartwright, 1953), although at the same time the surface area of the tissue may increase by more than 200 per cent. On the other hand, some results of experiments by Burström (1942) indicate that a close relationship exists between the changes in surface area and the amount of osmotically active substances within the cells of excised wheat roots.

There is also an increase in the concentration of ions and other substances within the vacuoles when cells are plasmolysed. The importance of internal solute concentration as a controlling factor in the absorption of mineral salts has been emphasized by a number of workers (Hoagland and Broyer, 1936; Humphries, 1951; Sutcliffe, 1952*a*), but the reason for it is not yet completely understood. In particular it has been difficult to distinguish between the effects of a high concentration of ions in the vacuole *per se* and indirect influences due to increased cell turgidity. With plasmolysed cells it is possible to alter the vacuolar concentration of ions without affecting turgidity, and a separation of these factors may therefore be attained.

Plasmolysed cells also differ from those in the normal condition in the quantitative relationship between the absorption of water and mineral salts. Usually the absorption of ions by a plant cell is accompanied by a certain uptake of water until it ceases to grow and is fully turgid. Subsequently a limited accumulation of solutes alone is possible until a maximum internal concentration is attained and absorption stops (Sutcliffe, 1952*a*). With plasmolysed cells the amount of water absorbed during recovery is probably greater than that which occurs in the normal cell for a given uptake of salts, owing to the absence of a wall pressure resisting its entrance. This may have an effect on the rate of ion accumulation since there is evidence (Commoner, Fogel, and Muller, 1943) that the absorption mechanisms for water and mineral salts by plant cells may be closely interrelated. Apart from the modifying influence which the entry of water exerts on the internal concentration of solutes, it is possible that if a considerable proportion of the water which enters a cell is metabolically transported by a mechanism such as that postulated by Goldacre (1952), it may compete with ions for the available valencies of the protein carrier molecules. These possibilities have been examined by controlling the rate of recovery of plasmolysed cells, and hence the rate of water absorption, to determine its effect on the uptake of potassium.

Finally it is known from the work of Bennet-Clark and Bexon (1943) that plasmolysis interferes with cell metabolism. They observed that when beet cells are plasmolysed in various media there is a rapid temporary increase in the rate of respiration, which is followed by reduction to a level which is generally lower than that of normal cells. In view of the dependence of ion accumulation on metabolic energy, an attempt has been made in the present investigation to correlate the effect of plasmolysis on absorption with the influence it exerts on respiration.

EXPERIMENTAL

Material. The material used in this investigation consists of disks of red beetroot tissue (*Beta vulgaris*, var. 'Crimson Globe'). Some of the advantages of this material for mineral salts absorption studies have already been discussed (Sutcliffe, 1952*a*). The presence of a coloured pigment in the vacuolar sap of the tissue made possible visual observations of the extent of plasmolysis during the course of the experiments. Furthermore, other investigations involving plasmolysed cells have already been made with this material (Bennet-Clark and Bexon, 1943; Myers, 1951).

Methods. The experimental material was prepared as has been described in a previous paper (Sutcliffe, 1952*a*), except that in the present investigation thinner disks (0·5 mm. instead of 0·75 mm.) were used to facilitate microscopic observations of the degree of plasmolysis attained with different experimental treatments. The tissue was generally used after it has been washed in aerated distilled water for about 7 days at 25° C., during which time a maximum absorptive capacity was developed. In one series of experiments, however, the disks were allowed to absorb ions during pretreatment by washing them in a solution of KCl instead of distilled water.

After the preliminary treatment the material was washed several times in fresh distilled water, dried superficially with filter paper to remove adhering liquid, and weighed in groups of 30 disks representing about 0·75 g. of tissue. Each batch of disks was then transferred to a 4-ml. aliquot of the plasmolysing or control medium contained in a 50-ml. wide-mouthed conical flask. This was provided with a centre well into which 0·3 ml. of a 10 per cent. KOH solution and a slip of filter paper were introduced to absorb CO_2 in connexion with oxygen uptake determinations which were made during the course of each experiment. Each flask was then closed by an adapter incorporating a Warburg-type manometer and attached to an apparatus by means of which it was shaken at a constant temperature of either 7° C. or 25° C. for 6 hours. During the experiments at 25° C. measurements of oxygen uptake were made by reading the manometers at 30-minute intervals. At the low temperature, where the amount of oxygen absorbed was small, a single reading only was taken at the end of each experimental period. The respiration results were later calculated from the data as μl. oxygen/g. fresh weight/hour.

At the end of 6 hours the absorption of potassium from the media was determined by flame photometry on a 3-ml. aliquot from each flask after dilution to 50 ml. with distilled water. From these results the absorption of potassium by the tissue was calculated and expressed as μg. K^+ absorbed/g. fresh wt. in 6 hours. A correction was applied where necessary to allow for dilution of the medium by water which left cell vacuoles on plasmolysis.

Three replicate flasks were set up for each treatment and the whole experiment was repeated twice, so that the absorption and respiration results presented below are each based on 6 separate determinations. Agreement between replicates in the same experiment was generally better than 5 per cent., and in separate experiments they did not differ by more than 10 per cent.

One experiment was designed to determine the effect of changing the rate of water movement into or out of plasmolysed cells on the rate of ion absorption. In this case groups of 30 disks were placed in 3-ml. aliquots of the plasmolysing medium, and during the course of absorption extending over 6 hours at 25° C. either 1 ml. of a more concentrated solution of the plasmolyticum or 1 ml. of 0·02 M. KCl was added 0·1 ml. at a time from a microburette at intervals of 30 minutes until the whole was added. In this way the osmotic pressue of the medium was made either to increase or decrease progressively during the course of an experiment independently of the absorption of ions by the tissue, and to cause corresponding changes in the water content of the material.

The nature and concentration of the plasmolytica. In all the experimental solutions the concentration of K^+ added in the form of KCl was maintained constant at 0·02 M. to avoid the complicating influence which concentration is known to exert on the rate of absorption of ions. The initial concentration of K^+ in the tissue, assuming that it is uniformly distributed, was estimated to be 0·05 to 0·07 M., so that in all cases the absorption of K^+ occurs against a considerable concentration gradient. The osmotic pressures of the various plasmolysing media were adjusted to the required values by the addition of another substance. Clearly the nature of the plasmolysing agent may be of considerable importance in determining the effect of plasmolysis, and to examine this a number of different plasmolytica were employed and their effects compared. The substances used were sucrose, glucose, sorbitol, calcium chloride, and magnesium sulphate. It is considered that these substances penetrate cells relatively slowly by comparison with KCl, and therefore caused only a small deplasmolysing effect during the experiments. Any metabolic influences which are exerted on the cells by the agents is expected to be different in each case, so that any effect which all produce in common at a plasmolysing concentration may be attributed to plasmolysis as such, and not to a secondary influence through an effect of the plasmolysing agent directly upon metabolism. In this connexion also, control experiments were arranged in which the various plasmolysing substances were added to the KCl solution at a high but non-plasmolysing concentration to distinguish direct metabolic effects unrelated to plasmolysis.

In all cases where calcium chloride was not itself the plasmolysing agent, this salt was added to the media, at a concentration of 0·002 M. to prevent any deleterious effect on the cells of unphysiologically balanced solutions of KCl.

A determination of the O.P. of the material was made at the beginning of each experiment by the method of 50 per cent. plasmolysis (Bennet-Clark, Greenwood, and Barker, 1936). At different times, with different material and different plasmolysing agents, values of 8–19 atm. were obtained. To cover this range, and to allow for various degrees of plasmolysis, solutions were prepared with O.P. of about 10, 15, 20, and 30 atm. Control experiments were conducted in a 0·02 M. KCl+0·002 M. $CaCl_2$ solution which has an O.P. of about 0·9 atm., and in solutions where the O.P. was raised to 5 atm. by addition of an appropriate amount of the plasmolysing agent.

No attempt was made during the present investigation to follow in detail the changes which occurred in the O.P. of the medium during the course of an absorption experiment through the uptake or metabolic utilization of the plasmolysing agent. It seems improbable that the O.P. of any medium altered appreciably through these causes during the 6 hours' experimental period. Microscopic observations of the degree of plasmolysis of the cells were made after, as well as before, the experiments, and it was found that this was not significantly altered during 6 hours at the low temperature. At 25° C., cells which were just plasmolysed in the 10-atm. media generally showed complete recovery at the end of an experiment, but in the media of higher concentration the tissue remained strongly plasmolysed throughout the experimental period. It may be noted that with unplasmolysed cells of this material the absorption of 1 mg. of KCl by 1 g. of tissue corresponds to an increase in the internal concentration of about 0·0125 M. and raises the O.P. of the cells by about 0·5 atm., assuming that the ions become uniformly distributed throughout the material and remain dissociated.

The degree of plasmolysis was only approximately the same with isotonic solutions of the different plasmolytica, but no attempt was made to examine in detail the small differences which were observed.

EXPERIMENTAL RESULTS

The results of a series of absorption experiments conducted at 25° C. with material of three different O.P.s are shown in Tables I to III. The tissues with an initial O.P. of 9 and about 13 atm. were subjected to washing in distilled water during the preliminary treatment, whilst that with an initial O.P. of about 19 atm. had been allowed to absorb KCl for 7 days before the experimental period.

It is clear from the data of Tables I–III that the effects of the different plasmolysing agents in each series of experiments are in general the same, and the results can therefore be summarized by the mean values indicated in the tables for each concentration of the medium. These data are represented diagrammatically in Fig. 1.

TABLE I

K^+ absorbed (μg./g. fresh wt.) by beet disks in 6 hours at 25° C., from media of different O.P. but containing KCl at a concentration of 0·02 M., when the initial O.P. of the tissue was about 9 atm.

Plasmolysing agent	O.P. of the tissue	O.P. of the medium					
		0·9	5	10	15	20	30
Sucrose . . .	9·2	1,340	1,785	990	1,105	1,220	1,135
Glucose . . .	8·3	1,475	1,820	950	1,215	1,055	1,105
Sorbitol . . .	9·7	1,385	1,590	1,065	980	1,080	960
$CaCl_2$ . . .	9·1	1,540	1,895	790	850	980	880
$MgSO_4$ . . .	8·9	1,505	1,675	960	1,190	1,285	1,045
Mean . . .	9·04	1,450	1,755	950	1,070	1,125	1,025

TABLE II

K^+ absorbed (μg./g. fresh wt.) by beet disks in 6 hours at 25° C., from media of different O.P. but containing KCl at a concentration of 0·02 M., when the initial O.P. of the tissue was about 13 atm.

Plasmolysing agent	O.P. of the tissue	O.P. of the medium					
		0·9	5	10	15	20	30
Sucrose . . .	12·1	1,270	1,690	1,715	820	1,140	1,010
Glucose . .	13·7	1,455	1,755	1,870	1,045	1,260	1,210
Sorbitol . .	12·3	1,465	1,805	1,795	980	1,065	1,105
$CaCl_2$ . . .	12·9	1,305	1,710	1,760	790	1,095	940
$MgSO_4$ . .	13·2	1,390	1,560	1,645	945	1,220	1,190
Mean . .	12·84	1,375	1,705	1,755	915	1,155	1,090

TABLE III

K^+ absorbed (μg./g. fresh wt.) by beet disks in 6 hours at 25° C., from media of different O.P., containing KCl at a concentration of 0·02 M., when the initial O.P. of the tissue was about 19 atm.

Plasmolysing agent	O.P. of the tissue	O.P. of the medium					
		0·9	5	10	15	20	30
Sucrose . . .	18·5	120	350	680	610	380	310
Glucose . .	19·3	85	425	590	640	210	220
Sorbitol . .	18·7	40	290	520	560	310	255
$CaCl_2$ . . .	19·3	65	220	305	280	190	110
$MgSO_4$ . .	18·9	110	480	710	740	380	310
Mean . .	18·94	85	355	560	565	295	240

The results show that all the plasmolysing agents stimulate the absorption of K^+ when they are present in the medium at a high but non-plasmolysing concentration. From Table I it appears that uptake was increased by between 12 and 33 per cent. when the O.P. of the medium was increased from 0·9 to 5 atm., the exact amount depending on the particular plasmolysing agent employed. On the other hand, a further increase in O.P. with this material caused the rate of absorption to be reduced. That this cannot be attributed directly to an increase in concentration of the medium is indicated by the fact that with material of higher initial O.P. absorption was promoted in both the 5- and 10-atm. media, as is shown by the data of Table II. The most striking stimulations of absorption were, however, observed with the high-salt tissue which had an initial O.P. of about 19 atm. The results of Table III show that the cells of this material absorbed only a relatively small amount of K^+ from the solutions of lowest O.P., but absorption was increased by more than 400 per cent. in the more favourable media with an O.P. of 5 atm. Absorption was further stimulated in the 10- and 15-atm. solutions in some cases to more than 600 per cent. of the controls.

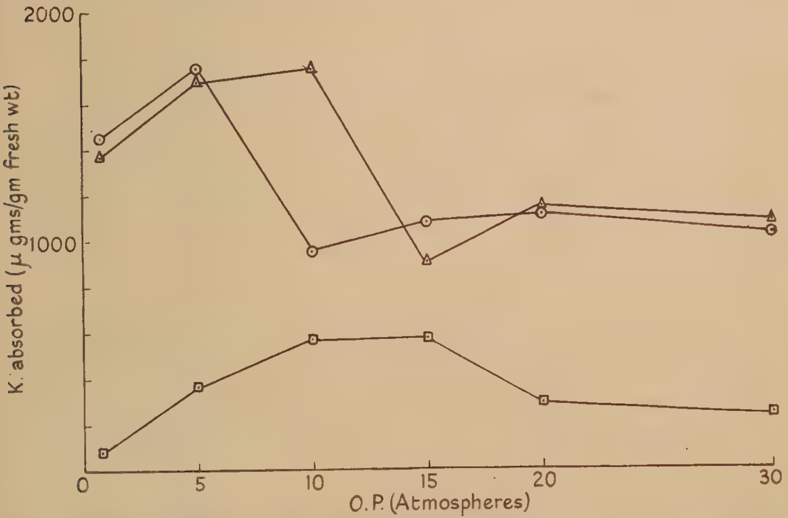

FIG. 1. Potassium absorbed by beet disks in 6 hours at 25° C. from media of O.P. shown as abscissae. Disks of 9-atm. internal O.P. (—⊙—), 13-atm. internal O.P. (—△—), 19-atm. internal O.P. (—⊡—).

Although uptake is stimulated by the plasmolysing agents when they are present in the medium at a non-plasmolysing concentration, it is evident that the effect of plasmolysis itself is to reduce the absorption of K^+. From the data of Tables I and II it may be calculated that uptake is reduced when plasmolysis occurs to between 66 and 84 per cent. of the amount of K^+

absorbed by the controls in the absence of the plasmolysing agents, and to between 52 and 67 per cent. of the maximum absorption attained in the media with a high but non-plasmolysing O.P. On the other hand, the results of Table III show that although the rate of absorption of K^+ with the high-salt material is reduced to below its maximum value upon plasmolysis, uptake continues more rapidly than in the controls with the media of lowest O.P. It may be noted from Tables I to III and from Fig. 1 that the degree of plasmolysis does not have a very profound effect on the absorptive capacity of the cells.

In order to understand more fully the influence which plasmolysis exerts on the absorption of K^+, determinations were made of uptake from the various media at a low temperature, namely 7° C., which causes metabolic absorption to be much reduced although physical uptake is probably unaffected. The results of an experiment performed at the same time, and with similar material to that which was recorded in Table I, are shown in Table IV.

TABLE IV

K^+ absorbed (µg./g. fresh wt.) by beet disks in 6 hours at 7° C. from media of different O.P., containing KCl at a concentration of 0·02 M., when the initial O.P. of the tissue was about 9 atm.

Plasmolysing agent	O.P. of the tissue	O.P. of the medium					
		0·9	5	10	15	20	30
Sucrose . . .	9·2	505	480	130	380	470	500
Glucose . . .	8·3	560	515	210	405	390	470
Sorbitol . . .	9·7	520	530	70	420	510	495
$CaCl_2$ . . .	9·1	610	555	245	315	465	530
$MgSO_4$ . . .	8·9	650	580	180	260	485	585
Mean . . .	9·04	570	530	165	355	465	515

When the data of Table IV are compared with those of Table I, it appears that the total uptake of K^+ in 6 hours at 7° C. from the 0·9-atm. media was about 40 per cent. of that at 25° C. With the 5-atm. media the proportion was rather less (about 30 per cent.) since although the total uptake of K^+ was stimulated by this treatment there was a small decrease in the amount of non-metabolically absorbed ions. Upon plasmolysis the uptake of K^+ at the low temperature was reduced still further. Absorption under these conditions was least from the 10-atm. media and increased somewhat as the degree of plasmolysis became more intense, so that the uptake of K^+ at 7° C. from the 30-atm. solutions was only slightly lower when mean values are compared than that of the material in the 5-atm. series.

The non-metabolic uptake of ions by plant cells depends on adsorption phenomena, exchange reactions, and diffusion. An attempt has been made to determine which of these several processes is mainly affected when non-metabolic absorption is reduced by plasmolysis.

Media were prepared with the non-electrolyte plasmolysing agents as described above, but KCl and $CaCl_2$ were omitted. The rate of diffusion of K^+ from the tissue was then determined by analysing the bathing solutions at the end of an experimental period of 6 hours. The experiment was conducted at 7° C. to reduce the metabolic reabsorption of ions. In the absence of cations in the external medium it was considered that the appearance of K^+ through exchange reactions was prevented. The results of this experiment are shown in Table V.

TABLE V

K^+ *released* (μ*g./g. fresh wt.*) *by beet disks in 6 hours at 7° C. into media of different O.P.*

Plasmolysing agent	O.P. of the tissue	O.P. of the medium					
		0·9	5	10	15	20	30
Sucrose . .	8·4	—	—	335	155	115	60
Glucose . .	8·9	15	10	360	175	105	45
Sorbitol . .	9·5	5	—	305	210	120	75

The results of Table V show that although there was no significant leakage of ions from unplasmolysed cells under the conditions of this experiment, potassium ions were released from plasmolysed tissue, and the effect was greatest at incipient plasmolysis. These observations are consistent with the view that the influence of plasmolysis in reducing the uptake of K^+ at 7° C. is exerted mainly, if not entirely, through an increased rate of leakage of ions from the cells.

It seems likely that the leakage of K^+ ions is promoted by plasmolysis more or less uniformly in all the cells of the tissue rather than by the damage of a certain proportion of them, since in the latter event leakage might be expected to be at least as great when the material is strongly plasmolysed as at the point of incipient plasmolysis.

Respiration. It is evident from a comparison of Tables I and IV that plasmolysis has an effect on the metabolic as well as the non-metabolic uptake of K^+, and in investigating the former a study was made of the influence of plasmolysis on oxygen absorption. The results of one of these experiments are shown in Table VI.

TABLE VI

Oxygen absorbed (μ*l./g. fresh wt./hr.*) *by beet disks placed in media of different O.P. at 25° C.*

Plasmolysing agent	O.P. of the tissue	O.P. of the medium					
		0·9	5	10	15	20	30
Sucrose. . .	9·2	84	127	138	123	112	101
Glucose . .	8·3	78	160	157	144	122	99
Sorbitol . .	9·7	70	81	118	109	98	86
$CaCl_2$ . . .	9·1	81	76	105	77	62	51
$MgSO_4$ . .	8·9	87	91	101	97	87	76

These data were obtained during the course of an absorption experiment the results of which are shown in Table I. The results of Table VI show that in contrast to the influence on absorption there are considerable differences between the effects of various plasmolysing agents on the rate of respiration.

It was observed that sucrose and glucose cause a large stimulation of respiration at the non-plasmolysing concentration, and a similar but smaller effect was observed with sorbitol. On the other hand, the presence of calcium chloride or magnesium sulphate in the medium did not influence the rate of oxygen uptake when the O.P. of the solution was 5 atm. Plasmolysis in sucrose or glucose solutions with an O.P. of 10 atm. did not cause any further change in the rate of respiration, but with the other plasmolytica oxygen uptake was increased when the O.P. of the medium was raised from 5 to 10 atm. Stronger plasmolysis resulted in a decrease in the rate of respiration from the highest values observed in each case.

A further series of experiments was designed to examine the causes of the respiration effects instigated by plasmolysis. It is known that extracts from beet cells stimulate the respiration of this tissue (Bennet-Clark and Bexon, 1943), and the possibility was investigated therefore that upon plasmolysis there is diffusion of respiratory active substances from the material. The investigation was carried out using $CaCl_2$ and $MgSO_4$ as plasmolysing agents since these substances do not themselves stimulate respiration at the non-plasmolysing concentration. Disks were plasmolysed in the 10-atm. media for 18 hours at 7° C., the material/medium volume ratio being double that which was used in normal absorption experiments. At the end of this time the liquid was decanted off and diluted with an equal quantity of 0·02 M. KCl. In this manner a medium was obtained with an O.P. of about 5 atm. containing KCl at a concentration of 0·02 M., and including any substances which may have diffused from the plasmolysed cells. The rate of respiration of beet disks in this medium was then compared with that which was observed in the normal 5- and 10-atm. media with the same plasmolysing agent. The results of the experiment are shown in Table VII.

TABLE VII

Oxygen absorbed (μl./g. fresh wt./hour) by beet disks at 25° C. in media of various O.P., in the presence and absence of diffusates from plasmolysed cells

Plasmolysing agent	O.P. of the medium					
	0·9	5	5 + diffusate	10	30 + diffusate	30
$CaCl_2$	84	86	122	115	89	91
$MgSO_4$	81	79	114	121	85	79

It is evident from the data of Table VII that the effect of plasmolysis on the rate of oxygen absorption in the 10-atm. media is to be attributed to a diffusion of respiratory active substances from plasmolysed cells, since respiration is stimulated in the non-plasmolysing 5-atm. media when the diffusate is present.

The results of Table VI show that although the rate of respiration of beet cells is stimulated at incipient plasmolysis, immersion of the tissue in a medium of higher O.P. causes a reduction in the uptake of oxygen. The cause of this phenomenon was investigated by a modification of the procedure described above, in which a medium with an O.P. of about 30 atm. was prepared containing a diffusate from plasmolysed cells. Tissue was plasmolysed in solutions of $CaCl_2$ and $MgSO_4$ with O.P.s of 10 atm. for 18 hours at 7° C., and the liquid was then decanted and diluted with an equal quantity of 0·02 M. KCl containing the plasmolysing agents at a concentration which was sufficient to increase the O.P. of the final solution to 30 atm. The rate of respiration of disks plasmolysed in these media was then determined and compared with that of disks in 30-atm. media with the same plasmolysing agent but in the absence of the diffusate. The results of this experiment are shown in the two right-hand columns of Table VII.

They show that the rate of respiration of strongly plasmolysed cells is not stimulated by those substances which cause a promotion of oxygen uptake by cells at incipient plasmolysis.

The possibility was then considered that the effect of plasmolysis on the metabolic absorption of K^+ may be attributed to the diffusion of an inhibitor of salt uptake from plasmolysed cells. To determine this, measurements of K^+ uptake were made at the end of the experiments in which the respiration data recorded in Table VII were obtained. The results of the absorption determinations are shown in Table VIII.

TABLE VIII

K^+ *absorbed* (*μg./g. fresh wt.*) *in 6 hours at* 25° *C. Other details as for Table VII*

Plasmolysing agent	O.P. of the medium					
	0·9	5	5 + diffusate	10	30 + diffusate	30
$CaCl_2$ . . .	1,570	1,805	1,895	1,115	1,005	1,085
$MgSO_4$ . .	1,455	1,743	1,840	1,210	1,140	1,165

The results of Table VIII show that the diffusate from plasmolysed cells has no inhibitory effect on the absorption of K^+, but on the contrary there is a small stimulation of uptake with the 5-atm. solutions.

The relationship between the absorption of water and K^+. To examine the relationship between water and salts uptake by plasmolysed cells an experiment was devised in which, on the one hand, the absorption of water was stimulated during the course of K^+ absorption by a progressive dilution of the external medium, and on the other the medium was allowed to become increasingly more concentrated, so that water was removed continuously from the tissue during the uptake of K^+. In one case the O.P. of the solution was decreased from 30 atm. to about 21 atm., and in the other case it was increased from 20 atm. to about 29 atm. The concentration of K^+ in the medium was maintained at about 0·02 M. throughout the experiment.

It is clear from the results of this experiment shown in Table IX that an alteration in the rate at which water enters or leaves plasmolysed cells does not affect the rate of K^+ absorption under the present experimental conditions.

TABLE IX

K^+ *absorbed* (*μg. g. fresh wt.*) *by beet disks plasmolysed in various media for 6 hours at 25° C. under conditions which facilitate either the uptake or remova of water from the tissue*

Plasmolysing agent	O.P. of the medium				
	0·9	20	20 → 29	30 → 21	30
Sorbitol . . .	1,480	1,150	1,210	1,165	1,085
$CaCl_2$	1,515	1,215	1,195	1,140	1,010
$MgSO_4$ . . .	1,360	1,005	1,105	1,040	965

DISCUSSION

The experimental results presented above show that all the plasmolysing agents employed in the present investigation stimulate the absorption of K^+ when they are present in the medium at a non-plasmolysing concentration This observation will be considered first before an attempt is made to analyse the effects of plasmolysis itself.

The influence of sucrose and glucose in promoting the rate of ion-uptake can be attributed to a metabolic influence in stimulating respiration, and through it the accumulation mechanism. The effect observed is not inconsistent with the hypothesis of Jacobson, Overstreet, King, and Handley (1950) that the absorption of salts depends upon the metabolic synthesis o carrier substances to which the ions become bound. The stimulation of K^+ absorption by sorbitol may be attributed to a similar influence since oxygen absorption was also increased with this substance, but it must be noted by comparison with the effects of sucrose and glucose that there is no direc relationship between the amount of metabolic stimulation and the increase i K^+ uptake. Sorbitol has a much smaller respiratory effect than either sucrose or glucose, and yet it is not much less effective in promoting absorption.

On the other hand, the two electrolytes used as plasmolysing agents promoted the uptake of K ions without having any significant effect on the rate of oxygen absorption by the tissue. An effect of multivalent ions, includin Ca^{++} and Mg^{++}, in stimulating the absorption of K^+ by barley roots has bee reported by Viets (1944). Although he also observed an effect of these ion on respiration, he claimed that the promotion of uptake may be due, not to direct influence on the oxidative mechanism supplying the energy for accumulation, but to an effect on the protoplasmic membrane. That a stimulatio of respiration is not essential for the increase of ion-uptake by $CaCl_2$ an

MgSO_4 is clear from the results presented above. Lundegårdh (1940) suggested that the effect of Ca^{++} in increasing the uptake of K^+ by wheat roots s due to an alteration of the electrical potential of the root surface which ncreases the degree of dissociation of membrane phosphatides on to which ations become adsorbed. It seems unlikely that this can be the causal factor n the present case since there appears to be no greater amount of K^+ absorbed rom the 5-atm. than from the 0·9-atm. media at low temperature. An alter-ative hypothesis is that proposed by Overstreet, Jacobson, and Handley 1952). They have suggested that Ca^{++} stimulates the absorption of K^+ by arley roots by facilitating the metabolic breakdown of a K^+ complex formed s an intermediary during absorption.

The possibility that another factor in addition to those considered above nay be responsible for the stimulating effects of the plasmolysing agents nust be considered. This is indicated by the relatively large stimulatory effect f sorbitol, which is metabolically rather inert, and by the fact that in some ases absorption is stimulated when the concentration of the plasmolysing gent is increased beyond the value at which an enhanced metabolic effect an be expected. This factor may be related to the decrease in turgidity of the issue which occurs when cells are placed in a medium of relatively high O.P. acques (1938) observed that the rate of absorption of ions by *Valonia* oenocytes is increased when the pressure exerted by the protoplasm on the nelastic cell-wall is artificially relieved. He suggested that the effect of ncreased turgidity in reducing uptake may be attributed to dehydration of he protoplasm which increases its resistance to the movement of ions. There s evidence from the present investigation against the specific mechanism roposed by Jacques since it is shown that absorption may be promoted in nedia which on account of the high O.P. might be expected to cause a reater dehydration effect than that exerted by the wall of a turgid cell. Other elated mechanisms, however, still remain as possibilities. It is possible, for xample, that the cell wall under pressure offers an increased resistance to liffusion so that ions reach the accumulation mechanism less easily when a ell is turgid, or there may be an increase in the thickness of the surface nembrane of the protoplast when it is forced into the pores of the wall by n increased pressure from inside, thus rendering the membrane more mpermeable.

Another factor which may be of some importance in promoting the absorp-ion of K^+ ions from solutions of high O.P. is that the molecules of the plasmo-ysing agent probably surround themselves by a thick shell of water molecules vhich thereby increases the effective concentration of K^+ in the medium. It as been shown with beet disks (Sutcliffe, 1952*a*) that the rate of absorption f K^+ is increased under the present experimental conditions when the oncentration of ions in the medium is increased beyond 0·02 M.

The effect of plasmolysis. Whatever the factors operative in promoting K^+ ptake when the O.P. of the medium is high, it is evident that in the absence f any complicating influences the rate of absorption should be maintained at the high level or even enhanced when plasmolysis occurs. In fact the data show that there is an inhibition, and some of the reasons for this will now be considered.

Part of the effect can be attributed, as has been shown above, to the leakage of ions from plasmolysed cells which reduces the net uptake of ions without directly affecting the accumulation mechanism. About 40 to 50 per cent. of the inhibition caused by plasmolysis in the 10-atm. media can be accounted for in this way, as can be shown by a comparison of the data of Tables I and V, but with respect to the more concentrated media the proportion is considerably less since in this case the amount of leakage is reduced whilst total absorption is only slightly increased. The higher rate of leakage of ions from cells at the point of incipient plasmolysis may be due to an increase in the area of protoplasmic surface available for outward diffusion when contact with the cell wall is lost (cf. Bennet-Clark and Bexon, 1943; Myers, 1951). When plasmolysis becomes more intense, leakage will tend to be reduced through a decrease in surface area, and an increase in the thickness or density of the protoplasts may be a contributory cause. These effects may be reversed to a certain extent by the greater internal concentration of ions which increases the activity gradient along which diffusion occurs, but evidently this effect is less efficacious than the other two in promoting the rate of leakage of ions from plasmolysed cells.

In addition to promoting leakage, plasmolysis also has an effect on the metabolic absorption of ions. This is shown by the data of Table X, which are derived from the determinations of total and non-metabolic absorption presented in Tables I and IV above.

TABLE X

K^- *absorbed* (μ*g. g. fresh wt.*) *by beet disks from solutions of different O.P. Calculated from data of Tables I and IV*

	O.P. of the medium					
	0·9	5	10	15	20	30
Total uptake . .	1,450	1,755	950	1,070	1,125	1,025
Non-metabolic uptake . .	570	530	165	355	465	515
Metabolic uptake .	880	1,225	785	715	660	510

Table X shows that metabolic as well as total uptake is progressively reduced as the degree of plasmolysis is increased. One of the factors which may inhibit the metabolic absorption of K^- by plasmolysed cells is the increased concentration of ions in the vacuoles. This effect is probably distinct from the influence of internal concentration in promoting the leakage of ions, since with normal cells absorption is reduced with increasing solute content although there is not an appreciable leakage in this case (Sutcliffe, 1952*a*). The influence of a high internal concentration in reducing the uptake of salts has been explained by Jacobson, Overstreet, King, and Handley

(1950) in terms of their hypothesis that uptake depends on the formation of complexes between ions and carrier molecules which later break down according to the reversible reaction:

$$HR+K^{+} \rightleftharpoons KR+H^{+}.$$

They have proposed that in the presence of a high vacuolar concentration of K^{+} the carrier is converted mainly to the KR state and little HR is available for the absorption of more cations from the external medium. With beet disks the absorption of K^{+} from a solution of KCl ceases when the internal concentration of the ion is 0·15–0·2 M. It may be predicted therefore on the basis of the above hypothesis that absorption by plasmolysed cells would be prevented when, through contraction of the vacuoles, the internal K^{+} concentration became greater than this. When beet cells are plasmolysed in media with an O.P. of 30 atm., it is likely that the concentration of K^{+} in the vacuoles exceeds this critical value since the initial concentration in this tissue is probably at least 0·06 M. (Sutcliffe, 1952*a*) and upon plasmolysis the volume of cell vacuoles is reduced to about 33 per cent. of its original size. Nevertheless cells plasmolysed in this way are still capable of absorbing K^{+} rather rapidly, and this must be attributed, if the above hypothesis is to be accepted, to a secondary effect of the plasmolysing medium in promoting either the synthesis of more HR than is normally available, or in facilitating the breakdown of the carrier/ion complex.

At the same time there are other changes as well as increasing internal concentration associated with plasmolysis which must be expected to influence metabolic absorption. One of these is the reduction in surface area of the protoplasts in contact with the external medium when cells are plasmolysed. It may be calculated, on the assumption that protoplasts in the plasmolysed condition are spherical, that cells which are just plasmolysed in a medium with an O.P. of 10 atm. will have the surface of the protoplasts reduced by about 37 per cent. when they are transferred to a medium with an O.P. of 20 atm. The data of Table X, on the other hand, show that metabolic absorption was about 13 per cent. lower from a 20- than from a 10-atm. medium. This indicates that there is not an exact relationship between surface area and the rate of metabolic absorption with plasmolysed cells. Such an observation becomes understandable if uptake depends primarily upon a limited number of 'absorption centres' which merely become more concentrated when the surface area of the protoplast is reduced. A hypothesis similar to this has been proposed by Brown (1952) to account for the constant rate of absorption of sugar and mineral salts by extending root segments as the surface area of the cells increases during growth.

With plasmolysed cells the position is complicated by changes which occur in the thickness and perhaps the density of the protoplasts. This may cause the absorption mechanism to become less accessible to ions from the external medium, and be one of the factors which are responsible for the inhibition of metabolic absorption by plasmolysed cells. Again, as in the cases of the influence of concentration in the vacuoles and surface area, it may be expected that the effect will be more pronounced when the degree of plasmolysis is increased.

Finally there is upon plasmolysis a reduction in the rate of respiration which may perhaps be related to the factors discussed above. It is possible that with a decreased surface area of protoplasts and increased thickness or density of the protoplasmic layer the rate at which oxygen diffuses to the respiratory centres is reduced, and this becomes a factor limiting respiration. The possibility that the level of respiration is determined by an internal factor is supported by the observation made above that the rate of oxygen absorption of strongly plasmolysed cells cannot be stimulated so effectively by the provision of substrates or respiratory active diffusates in the external medium. At least a part of the reduction in the rate of metabolic K^+ absorption by cells upon increasingly stronger plasmolysis is probably attributable directly to a reduction in the available energy when oxygen absorption is decreased.

By an interaction of various factors discussed here it is possible to account for some of the inhibitions of ion absorption observed, and in particular to explain the differences between weakly and strongly plasmolysed cells in this respect. But it is also evident that the effects of plasmolysis cannot be accounted for entirely in this way. This is particularly obvious in the case of cells at about the point of incipient plasmolysis, when the effective surface area of the protoplasts is possibly greater than it is when the cells are in the normal condition. Moreover, the thickness of the protoplast and the internal concentration of ions may not be appreciably altered, whilst the rate of respiration becomes high. Amongst various other factors which may further inhibit absorption by plasmolysed cells, two may be rejected from the evidence presented above. It has been shown in the first place that the increased absorption of water probably does not affect the rate of K^+ uptake, and secondly the absorption of K^+ is not reduced by the leakage of an inhibitor substance from tissue in the plasmolysed state. Furthermore, the possibility that accumulation is reduced by an increased resistance of the cell wall to diffusion when contact with the protoplast is lost is unlikely, since Bennet-Clark and Bexon (1946) have shown that in onion epidermal cells there is no detectable difference between the compositions of the external medium and intramural, extraprotoplasmic fluid following plasmolysis.

A remaining possibility is that plasmolysis causes temporary changes in the protoplasts analogous to a 'wound response' which prevent the transfer of metabolic energy to the accumulation mechanism in the manner of dinitrophenol inhibition (Robertson, Wilkins, and Weeks, 1951), but evidence to support or reject this hypothesis is as yet lacking.

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The Assimilation of Nitrogen from Ammonium Salts and Nitrate by Fungi

A. G. MORTON AND ANNE MACMILLAN

Butterwick Laboratories, Imperial Chemical Industries, Ltd.

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SUMMARY

1. The assimilation of inorganic nitrogen by *Scopulariopsis brevicaulis* and some physiologically similar species has been studied. Their failure to assimilate completely from ammonium sulphate has been shown to be due to the fall in pH of the medium induced by the initial uptake of ammonia.

2. Complete assimilation of ammonia takes place in the presence of the neutral salts of each of thirteen organic acids investigated. The organic acids act primarily through their buffering effect which prevents or slows down the fall in pH. They are not specifically required for ammonia assimilation by these fungi and can be effectively replaced by certain inorganic buffers.

3. The influence of several external factors on the rate of assimilation of ammonia, nitrate, and nitrite has been studied in *S. brevicaulis*. In corresponding conditions the mycelium assimilates ammonia more rapidly than nitrate over a wide range of conditions.

4. Ammonia, even in very low concentration, completely suppresses nitrate assimilation when both sources of nitrogen are present together. Nitrite, however, is assimilated simultaneously with ammonia. It is therefore concluded that ammonia blocks the reduction of nitrate to nitrite by the fungus.

5. The suppression of nitrate assimilation in the presence of ammonia is common to many mould fungi besides *S. brevicaulis*, and is believed to have adaptive significance in natural habitats.

6. The nitrate-reducing and assimilating system is formed, even when *S. brevicaulis* is grown in complete absence of nitrate (ammonia medium with organic acid). It comes into action rapidly when the inhibiting effect of ammonia is removed. Similarly, nitrate-grown mycelium is capable of assimilating ammonia at maximal rate without any adaptive lag.

INTRODUCTION

FUNGI require more or less complex organic compounds as the source of carbon for growth, but are generally capable of using simple inorganic compounds of nitrogen. For most fungi ammonium salts are a suitable source of nitrogen, and many, but not all, of them can use nitrate as well. Fungi which grow on inorganic nitrogen are also capable of using various amino-acids, proteins, and other organic nitrogen compounds. Only a relatively small number of fungi appear to require organic nitrogen and to be incapable of using inorganic nitrogen, and these are usually specialized parasitic organisms.

The experiments reported here were designed to follow the course of assimilation by mould fungi of nitrogen from inorganic sources, in the first place from ammonium salts and nitrate, and to determine the influence of external factors on this process. The starting-point was provided by the

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observations of Brian, Curtis, and Hemming (1947), who showed the existence of an unusual pattern of nitrogen nutrition in certain species of fungi. They found that *Myrothecium verrucaria*, with glucose as carbon source, grows readily when the nitrogen source is ammonium tartrate, but scarcely at all when this is replaced by ammonium sulphate. Normal growth takes place on ammonium sulphate, however, in the presence of any one of a number of organic acids, added as neutral salts (potassium or sodium). On ammonium nitrate growth is as poor as on ammonium sulphate, although the fungus grows very well on potassium nitrate, from which nitrogen is vigorously assimilated. Growth on ammonium nitrate can be promoted by the addition of organic acids, as in the case of ammonium sulphate. Several other fungal species, including a strain of *Scopulariopsis brevicaulis*, showed essentially the same pattern of nitrogen nutrition. Brian, Curtis, and Hemming considered that in these fungi the assimilation of nitrate is somehow blocked by ammonia, which is therefore used preferentially, and that for the continued assimilation of ammonia one of a number of organic acids is required. It was decided to use these organisms as experimental material, using other species for comparison as necessary. Preliminary experiments revealed no essential difference in nitrogen nutrition between *Myrothecium* and *Scopulariopsis*. Since *Scopulariopsis* is more easily grown on synthetic media it was used for most of the experimental work.

Throughout this paper the terms ammonia and nitrate are used in a general sense to mean ammonia-nitrogen and nitrate-nitrogen.

MATERIAL

The following fungi have been used in the course of this investigation. Reference numbers in brackets refer to the Butterwick Culture Collection: *Alternaria solani* (Ell. and Mart.) Jones and Grout (408); *Aspergillus nidulans* (Eidam) Winter (598); *A. niger* v. Tieghem (32); *A. oryzae* (Alb.) Cohn (265); *A. repens* (Cda.) de Bary (560); *Cladosporium herbarum* Link (773); *Diplodia natalensis* Pole Evans (832); *Fusarium graminearum* Schwabe (30); *Mucor ramannianus* Moeller (645); *Myrothecium verrucaria* (Alb. and Schw.) Dim. (173); *Penicillium chrysogenum* Thom (65); *P. griseofulvum* Dierckx (375); *Phycomyces blakesleeanus* (+) var. *piloboloides* Burgeff (824), (825); *Ph. blakesleeanus* (+) Burgeff (826); *Ph. blakesleeanus* (Z—) Burgeff) (827); *Scopulariopsis brevicaulis* (Sacc.) Bainier (55), (55/OS/A1); *Trichoderma viride* Pers. ex Fr. (211).

The original material of *S. brevicaulis* (No. 55, Butterwick Collection) grows mainly superficially in liquid culture and does not flourish in shake culture. This material apparently contained several slightly differing strains, and in the course of this work one strain (No. 55/OS/A1) was isolated therefrom. This strain differs in its more submerged habit and in its ready growth in shake culture, and was used in most of the later experiments. No physiological differences with respect to nitrogen metabolism have been observed between the two strains, which are not, therefore, separately designated.

EXPERIMENTAL METHODS

Two main types of experiment have been used in this work. In the first place, the fungus has been grown for long periods in surface culture, starting from a small initial inoculum. Secondly, the uptake of nitrogen by the fungus was studied in defined conditions for periods of a few hours.

Culture experiments. The fungus is inoculated into a liquid medium dispensed in 100-ml. conical flasks, plugged and sterilized. Except in experiments which are specially mentioned the basal composition of the culture medium was not varied. It contained 5 per cent. glucose, 0·1 per cent. KH_2PO_4, 0·05 per cent. $MgSO_4.7H_2O$, with the addition of the trace-elements iron, copper, zinc, manganese, and molybdenum. To this basal medium different sources of nitrogen and other components were added as required. After inoculation the flasks were incubated at 25° C.

Replicate flasks were taken for analysis initially and at daily or longer intervals. For quantitative experiments exactly the same amount of nitrogen in the appropriate form was contained in each flask initially, and the initial volume of culture medium was the same in each flask (usually 20 ml.). The culture medium from each flask was made up to a standard volume with distilled water before carrying out analyses, in order to allow for evaporation during the experiment.

The normal level of carbohydrate in the basal medium is more than sufficient to allow complete assimilation of the nitrogen supplied.

Short-period uptake experiments. A suspension of fungal mycelium was used in a suitable medium maintained at 25° C. during the experimental period. The mycelial suspension was contained in flasks vigorously shaken to maintain an adequate supply of oxygen. By withdrawing aliquot samples for analysis at intervals the uptake of nitrogen from the medium can be followed for periods up to 6 hours. Longer periods have not been used since it is impracticable in this type of experiment to maintain completely aseptic conditions, and beyond 6 hours the risk of bacterial contamination becomes appreciable. The possible occurrence of bacteria in significant numbers was always carefully checked and only very rarely has it been necessary to reject experiments for this reason.

The composition of the medium, although varied for experimental purposes, was essentially the same as the medium used for culture experiments. It normally contained glucose (1 per cent.) and a nitrogen source at the same concentration as in culture medium, dissolved in 0·07 M. potassium phosphate buffer (pH 6·5–7·0).

Young mycelium (3–6 days old), at the stage of active growth and nitrogen assimilation, was used for experiment since this is the stage of most immediate interest.

As experiments were nearly always carried out in still culture it seemed preferable to use mycelium from this source also for the short-period experiments. The mycelium was broken up into a fairly homogeneous suspension by means of a high-speed blendor of the Waring type. This blendor was specially designed and constructed in these laboratories to enable mycelium to be broken up in aseptic conditions (for details see Moore and Mason, 1951). After blending, the mycelium was rapidly washed with distilled water on a sintered-glass filter and was used immediately for experiment. Mycelium was also used without blending, being merely cut up sufficiently with a stainless steel knife to give a reasonable suspension, and washed on a filter before being used. Replicates of blended and untreated mycelium show comparable rates of nitrogen uptake when the period of blending is less than 30 seconds. Many experiments have been repeated also with mycelium from shake culture: the general behaviour of this material does not differ from that of mycelium of comparable age from still culture.

In these experiments the loss of inorganic nitrogen from the medium was measured. Determinations of free ammonia and nitrate in the mycelium show that the level of inorganic nitrogen does not change appreciably during the experimental period. These results will be reported in detail in a separate publication dealing with the entry of ammonia and nitrate into the mycelium. It is sufficient to note here that the disappearance of inorganic nitrogen from the medium may be taken as a real measure of nitrogen assimilation by the fungus.

The amount of nitrogen taken up by the fungus is expressed as mg. N/g. initial dry weight. The difference between nitrogen uptake in duplicate treatments of the same batch of mycelium shows a standard deviation of 0·54 mg. N/g. dry matter. For practical reasons it was not possible to duplicate treatments in most experiments. Differences of less than 2 mg. N/g. dry matter are assumed to be of doubtful or of no significance.

Analytical methods. All analyses (except pH) were made in duplicate or triplicate and the results are given as the mean. Individual determinations rarely differed by more than 3 per cent. from the mean.

pH measurements were made on a 'Cambridge' bench pH meter. *Dry matter* was weighed after washing the mycelium free from culture medium and drying at 92° ± 2° C. overnight. *Ammonia* determinations were made in Conway units (Conway and Byrne, 1933) as described by Conway (1947), with 2 per cent. boric acid as absorbent, but using a weaker alkali (0·5 N. KOH) and leaving the units overnight before titration with 0·5 N. HCl. For samples containing less than 0·03 mg. NH_3, 0·02 N. HCl was used as absorbent and was transferred quantitatively to 10-ml. volumetric flasks and Nesslerized (Koch modification, Koch and Hanke, 1948). The colour was read in a Hilger Spekker with a violet filter (Hilger O.B. 1) and compared with a standard (recovery 90–99 per cent.). *Nitrate* was determined after reduction to ammonia with 0·1–0·2 g. Devarda's alloy in the outer chamber of Conway units using 0·5 N. KOH acting overnight. Pre-formed ammonia and Devarda's alloy blank must be subtracted. Nitrite if present is also reduced to ammonia by Devarda's alloy but may be destroyed by addition of 0·1 ml. of 10 per cent. sulphamic acid to the sample in the Conway unit 5 minutes before adding the alloy and alkali. *Nitrite* was determined with Devarda's alloy in the same way as nitrate. When both nitrate and nitrite are present the latter is calculated from the difference in titration with and without sulphamic acid treatment. Small quantities of nitrite (below 5 μg. N/ml.) were estimated colorimetrically with the α-naphthylamine reagent of Parker (1949).

EXPERIMENTAL RESULTS

Factors affecting nitrogen assimilation. The pattern of nitrogen nutrition of *Scopulariopsis brevicaulis* has already been outlined. Quantitative data are presented in Table I which summarize the results of many long-period culture experiments.

The fungus is evidently capable of assimilating nitrogen from ammonium sulphate. Some 15–20 per cent. of the ammonia initially present is assimilated in the first 3 or 4 days, but no more is assimilated by the fungus even when the period of culture extends to 40 days or more. The addition of the potassium (or sodium) salt of an organic acid permits in every case complete assimilation of the ammonia, although the final dry weight of the fungus shows a certain dependence on the nature of the organic acid added.

TABLE I

Growth of S. brevicaulis on different nitrogen sources (with glucose)

Nitrogen source .	KNO_3	NH_4NO_3	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$ + tartrate	$(NH_4)_2SO_4$ + malate
Mean dry matter (mg./flask) . .	200	18	12	160	225
Nitrogen assimilated (% of initial) .	100	15–20	15–20	100	100
Range of final pH .	7·0–8·1	3·0–3·9	2·7–3·8	3·4–4·4	7·5–8·6

On ammonium nitrate the fungus behaves as on ammonium sulphate. There is a small initial uptake of ammonia, after which no more nitrogen is assimilated. Addition of the salt of an organic acid to the ammonium nitrate medium allows the utilization of all the nitrogen, both ammonia and nitrate.

The pattern of nitrogen nutrition revealed in culture experiments shows considerable stability, indicating that it is an expression of the fundamental physiology of the organism. Minor changes in cultural conditions, such as solidification of culture media with agar, separate sterilization of the components, sterilization by Seitz filtration in the cold instead of by autoclaving, do not alter the results. The addition of a supplement containing biotin, pyridoxin, aneurin, nicotinic acid, and inositol did not affect nitrogen utilization.

It was thought that a higher concentration of potassium might be required in the presence of ammonium salts, since ammonium ions might tend to displace potassium ions at internal sites in the protoplasm. Increasing the concentrations of potassium in the medium, however, had no effect on the assimilation of nitrogen from ammonium sulphate or ammonium nitrate.

In short-period experiments the mycelium of *S. brevicaulis* was found to take up ammonia for several hours. The result of a typical experiment appears in Table II. Mycelium, previously grown in nitrate medium, was suspended in 0·07 M. phosphate buffer (pH 6·9) containing 1 per cent. glucose and 0·2 per cent. ammonium sulphate and was shaken to ensure access of oxygen. There is steady uptake during 5 hours and a total assimilation of 18 mg. of NH_3-nitrogen per gramme initial dry matter of mycelium.

TABLE II

Uptake of ammonia by S. brevicaulis shaken in phosphate buffer with glucose and ammonium sulphate

Time from start (hours)	pH of medium	Uptake of ammonia by mycelium (mg. N/g. dry matter)
0	7·6	—
0·5	6·6	3·2
1·0	—	5·8
3·5	6·1	12·6
5·0	6·0	18·3

Other experiments have demonstrated nitrogen uptake at almost constant rate for over 7 hours in these experimental conditions. It has been shown that the rate of uptake is not affected by variations in glucose concentration between 0·5 and 2 per cent., or by variations in ammonium sulphate concentration between 0·1 and 0·4 per cent. Shaking in air ensures an adequate supply of oxygen, for the rate of nitrogen uptake is not significantly increased by bubbling pure oxygen through the medium.

No assimilation of nitrogen (nitrate, nitrite, or ammonia) occurs in the absence of oxygen. In the absence of glucose there may be a significant uptake of nitrogen in the first hour only. The mycelium evidently does not normally contain sufficient mobilizable reserve materials to maintain nitrogen assimilation in the absence of external carbohydrate.

It should be made clear that no organic acid salt was added in these experiments. Ammonia uptake clearly proceeds vigorously at least for several hours when no organic acid is present. Experiments on the role of organic acids are described later.

Rate of nitrogen uptake and the pH of the medium. Culture experiments indicate that *Scopulariopsis brevicaulis* grows best when the pH of the medium lies between 6·0 and 7·0, and culture media were adjusted initially to pH 6·5 as closely as possible before inoculation. The actual pH as determined for a particular experiment lies in the range between 6·0 and 7·0, and unless otherwise stated, rates of nitrogen uptake by the mycelium have been measured in this pH range.

The results of a considerable number of experiments with different batches of *Scopulariopsis* on different occasions are collected in Table III. The rate of uptake of ammonia is as a rule considerably higher than the rate of uptake of nitrate in comparable conditions. The mycelium used was, in every case, taken from a surface culture, 3–5 days old, growing vigorously on nitrate medium. Since the mycelium was assimilating nitrate immediately prior to the experimental period, it would be expected to be fully adapted to the assimilation of nitrate but not necessarily to the assimilation of ammonia. It is therefore interesting to find that the mycelium appears also to be fully adapted to ammonia assimilation, for it not only switches over without lag but proceeds to take up ammonia about twice as rapidly as it takes up nitrate in corresponding conditions. The term 'adaptation' is used here to mean the quantitative adjustments in the activity of various enzyme systems rather than acquisition of new enzymes in response to external conditions.

TABLE III

Rate of assimilation of ammonia and nitrate nitrogen by S. brevicaulis at pH 6·5–7·0

Treatment	Mean rate as mg.N/g. dry matter/hour	Range	Number of observations
Short exposure:			
Ammonia-nitrogen	3·77±1·21	1·6–7·5	33
Nitrate-nitrogen	1·50±0·37	0·9–2·2	21
Long exposure:			
Ammonia-nitrogen	3·52±2·10	1·4–7·0	9
Nitrate-nitrogen	1·46±0·61	0·8–2·0	3

A comparison may be made between rates of nitrogen uptake in short-period experiments and the rates observed in long-period culture experiments, also given in Table III. These rates are for 3- to 5-day old mycelia and are calculated for observations at 24-hour intervals. The very close agreement in the results quoted is probably fortuitous and even slightly misleading, since comparison is complicated by differences in experimental conditions. However, the general agreement between the two estimates of nitrogen uptake strengthens the view that the short-period experiments can be a guide to the normal behaviour of the organism.

The difference in the rates of uptake of ammonia and nitrate at pH 6·5–7·0 led us to investigate rates of uptake over the physiological pH range. Replicate samples of the same batch of mycelium, grown on a nitrate medium, were compared for uptake of ammonia and nitrate at each selected pH. All the determinations could not be done on a single batch, but inoculations were made on successive days and each batch of mycelium was thus the same age at the time of the experiment.

The results are shown in Fig. 1. Except at the lowest pH the rate of uptake of ammonia is greater than that of nitrate over the whole range. At pH 3·5 the rate of uptake of both forms of nitrogen is barely significant, and this value seems to represent the lowest pH at which *Scopulariopsis* is capable of growth at all. This has been confirmed by culture experiments in which the media were initially adjusted to pH 3·5. In these conditions *S. brevicaulis* failed to grow either on nitrate medium or on ammonia medium containing an organic acid salt.

The divergence between the rates of uptake of ammonia and nitrate becomes more marked in the higher pH range. Whereas nitrate uptake reaches its maximum rate at about pH 6·0, ammonia uptake evidently reaches its maximum above pH 7·0. Determinations have not been extended sufficiently far above pH 7·0 to find out when the rate of ammonia uptake begins to fall off.

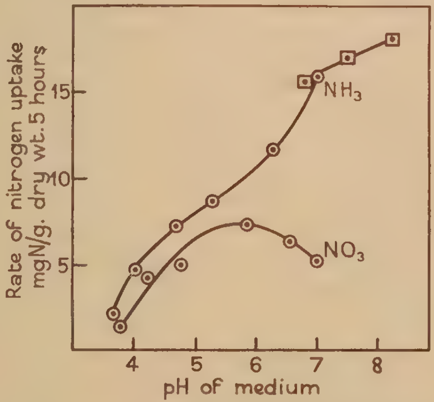

FIG. 1. *S. brevicaulis;* rate of uptake of ammonia and nitrate in relation to pH of medium. –⊙– O.I.M. citrate phosphate buffer –⊡– O.I.M. pyrophosphate buffer (separate experiment).

The high rate in this pH range is due to uptake by the mycelium and not to loss of gaseous ammonia to the atmosphere, as control experiments show.

The rate of uptake of ammonia thus appears to be greater than that of nitrate over the whole pH range. Dry matter accumulation is also initially more rapid on ammonia medium (with organic acid) although the final dry matter may be as high or higher on nitrate medium.

In the assimilation of nitrate by fungi the first recognizable reduction product is nitrite. This is shown by the appearance of some nitrite in the medium during nitrate-assimilation, and is supported by the fact that many fungi, including *Scopulariopsis*, grow normally when nitrogen is supplied as nitrite. Since nitrite does not normally accumulate in the medium it must be assimilated at about the same rate as nitrate. In short-period experiments at pH 6·5–7·0 young nitrate-grown mycelium of *Scopulariopsis* is found to take up nitrite at the rate of 1·2 mg. N/g. dry matter/hour, which compares closely with the rate of nitrate uptake in the same conditions.

Organic acids and nitrogen uptake. The experiments of Brian, Curtis, and Hemming (1947) with *Myrothecium* and our own results with *Scopulariopsis* show that the effect of various organic acids on nitrogen nutrition is exclusively connected with the assimilation of ammonia. If nitrogen is supplied as nitrate or nitrite, or as glycine, glutamate, aspartate, or as a mixture of amino-acids as in casein hydrolysate, then complete assimilation takes place with glucose alone. With ammonia nitrogen (ammonium sulphate or ammonium nitrate) maximum growth of the fungus, equivalent, that is, to growth on nitrate, is only attained if an organic acid (as neutral salt) is added in addition to glucose.

There seem to be three possible explanations of the 'organic acid effect'. (*a*) These fungi may possess a specific requirement for certain types of organic acid possibly to provide keto-acids in order to be able to assimilate ammonia, as suggested by Brian, Curtis, and Hemming (1947) for *Myrothecium*. (*b*) Organic acids may operate simply through an effect on the pH drift of the medium during growth. Since the medium is only very slightly buffered, the assimilation of ammonia from ammonium sulphate results in a rapid fall in pH, which the presence of an organic acid in salt form may counteract. (*c*) Organic acids may prevent formation of toxic substances in the medium during ammonia assimilation.

The experimental evidence which follows excludes the first and third hypotheses, and shows definitely that the effect of organic acids on growth of *Scopulariopsis* in ammonia media is primarily an effect on the pH drift of the medium.

The behaviour of the fungus with a number of organic acids is illustrated by a culture experiment (GNM 38) in which 0·075 M. formic, acetic, pyruvic, lactic, succinic, fumaric, *dl*-malic, *d*-tartaric, and oxalic acids as Na or K salts at pH 6·5 were added as supplements to both ammonium sulphate and potassium nitrate media.

The results on nitrate show no marked effect of organic acids on either growth or pH drift with this nitrogen source. On ammonia medium, however, the effect of the organic supplements on growth of the fungus is very pronounced (Table IV). Results of an earlier less complete but similar experiment (GNM 32) have been included.

TABLE IV

Effect of organic acid supplements on final dry weight (mg./flask) of Scopulariopsis brevicaulis in ammonia medium

Organic acid supplement	Exp. GNM 38 (20 days)	Exp. GNM 32 (17 days)
None	5	5
Formate	215	—
Acetate	210	80
Pyruvate	85	60
Lactate	55	—
Succinate	270	170
Fumarate	255	205
dl-Malate	245	—
d-Tartrate	240	110
Oxalate	100	—
Mean (for media with acid supplement)	185	125

The drifts of pH in the ammonia media are shown in Fig. 2. In the unsupplemented medium the pH falls rapidly to 3·5–4·0 in the first 3 days, after which growth ceases. The organic acids appear to form two groups. With lactate, pyruvate, oxalate, and tartrate, the pH falls steadily but more slowly than in unsupplemented medium, remaining above 4·0. With the remaining organic acids the pH does not fall below 5·0 and tends to rise in the later stages. However, succinate, fumarate, malate, and acetate frequently cause the same type of pH change as tartrate without any final rise. The reason for these differences in pH drift is not known.

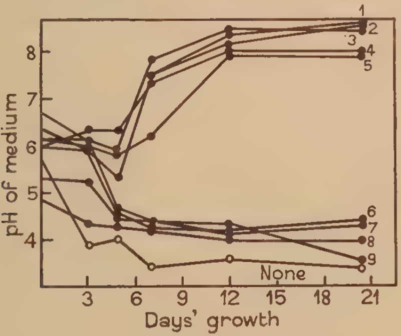

FIG. 2. *S. brevicaulis;* drift of pH in ammonia medium with organic acid supplements. 1 Malate, 2 Fumarate, 3 Formate, 4 Acetate, 5 Succinate, 6 Tartrate, 7 Pyruvate, 8 Lactate, 9 Oxalate.

The significant feature is the effect of organic acid salts on pH drift in the first stages of growth. Whereas in ammonia medium the pH falls to a level known to inhibit growth of *Scopulariopsis brevicaulis*, the presence of an organic acid salt causes the pH to fall much more slowly from its initial value (6·0–6·5) and even after 12 days it is still above 4·0.

In addition to the acids already mentioned, glycollic, gluconic, α-keto-glutaric, maleic, and citric acid have been found effective. The general effect of an acid supplement in arresting the fall of pH is clear. The lack of specificity in the response to different organic acid salts suggests that they function primarily by maintaining a favourable reaction in ammonia medium.

Short-period uptake experiments with young mycelium support this view. Experiments already quoted show that mycelium takes up ammonia steadily for several hours with no other carbon source but glucose. The addition of several organic acids does not affect the rate of ammonia uptake by the mycelium. Furthermore, there is no uptake of ammonia when glucose is omitted and various organic acids are added as the sole carbon source (Table V). In these experiments of relatively short duration, carried out in buffered solution, the pH change, even in the absence of an organic acid salt, is not sufficient to affect the rate of ammonia uptake significantly. Thus at suitable pH and for periods up to at least 5 hours, *S. brevicaulis* does not specifically require organic acids for the assimilation of ammonia, and the rate of assimilation is not increased in the presence of organic acids.

Results with inorganic buffers (Table VI) show that these substances cause considerable increases in growth of *S. brevicaulis* in ammonia medium, the highest yield (with calcium carbonate) equalling that on nitrate medium. The pH drifts in presence of the various inorganic supplements indicate that they do not all buffer the medium to the same extent, but in every case the pH is maintained at a higher level than in unsupplemented ammonia medium for at least the first 4 days. Ammonia was found to be completely assimilated in the presence of sodium bicarbonate, calcium phosphate, and other inorganic buffers.

TABLE V

Effect of organic acids on rate of uptake of ammonia (mg. N/g. dry weight/5 hours) by young mycelium of S. brevicaulis

Organic acid	Glucose only	Glucose + acid	Acid only	No carbon source
Formate	15·9	11·6	3·0	2·0
Acetate	9·2	9·1	2·7	2·0
Lactate	13·1	9·9	0·2	−0·9
Pyruvate	17·4	—	2·3	1·7
dl-malate	23·6	20·4	3·4	3·1
d-gluconate	12·0	11·8	2·3	1·2

TABLE VI

Growth of S. brevicaulis on ammonia medium in presence of inorganic buffers

Supplement	pH of medium				Dry matter (mg./flask)		
Days:	Initial	4	7	13	4	7	13
None	6·2	3·7	3·8	3·6	4	7	6
$CaSiO_3$*	6·1	4·9	4·0	2·7	85	100	111
$Ca(H_2PO_4)_2$*	5·9	5·8	4·4	4·5	65	—	100
$CaCO_3$*	7·8	7·7	7·3	7·3	110	120	160
$Na_2B_4O_7$ (0·03 M.)	6·5	7·0	5·9	5·6	60	80	115
$NaHCO_3$ (0·03 M.)	8·2	7·0	3·7	—	40	125	—

* Solid salt present in the medium.

In a further experiment the fungus was allowed to grow on unsupplemented ammonia medium. After 5 days the pH had fallen to 3·5–3·6, growth was very slight, and less than 5 per cent. of the total nitrogen had been assimilated. To some of the culture flasks an addition of 2 ml. of sterile 5 per cent. sodium succinate was made bringing the pH to 6·1. Other flasks were brought to pH 7·4 by addition of sterile sodium bicarbonate solution. Flasks which received no addition showed little further growth and the dry weight of the fungus at 18 days was 45 mg. per flask; those receiving succinate and bicarbonate showed vigorous growth and dry weights at 18 days were respectively 195 mg. and 160 mg. per flask, comparable with dry matter production on nitrate medium. Thus failure of the organism to grow in unsupplemented ammonia medium cannot be due to accumulation of toxic substances (other than hydrogen ions), and must be connected with the low pH which arises.

The effect of organic acids may be direct physical buffering where the acid is not utilized by the fungus, but an acid which is assimilated by the fungus at about the same rate as the ammonia would also largely prevent a fall in pH (by metabolic buffering) even if it had no physical buffering power. A combination of both types of buffering is possible with the same acid.

The primary effect of organic acids on the growth of *S. brevicaulis* on ammonia depends on their influence on pH drift. Experiments, not described in detail, indicate that with malate and several other acids this is due to physical buffering, with acetate and tartrate metabolism accompanies physical buffering, whilst the effect of pyruvate depends only on its metabolism by the fungus.

Some of the experiments described above were repeated with *Myrothecium verrucaria*, which was found to behave similarly to *S. brevicaulis*. In particular, *M. verrucaria* grew normally on ammonia in the presence of several inorganic buffers. In *Myrothecium*, as in *Scopulariopsis*, organic acids are thus concerned with pH drift, and are not specifically required for ammonia assimilation.

Many fungi which differ from *Scopulariopsis* and *Myrothecium* in growing moderately well on ammonia medium, nevertheless show improved growth with the addition of organic acid salts. Experiments with *Penicillium griseofulvum*, *P. chrysogenum*, *Cladosporium herbarum*, *Alternaria solani*, and other species which behave in this way have shown that the ameliorating effect must be ascribed to the influence of the organic acids on pH drift. Thus *Scopulariopsis* and *Myrothecium*, which at first sight seem very distinct in their ammonia nutrition, differ from many other fungi essentially only in their greater sensitivity to low pH, and hence also by their very marked response to organic acids.

We have also examined the behaviour of *Mucor ramannianus* and *Phycomyces blakesleeanus*, as these two species are claimed by Leonian and Lilly (1940) to require organic acids.

Mucor ramannianus grows moderately well on ammonia medium without supplement, but growth is markedly increased by inorganic buffers and organic acids. There does not seem to be evidence of any primary effect of the latter beyond their influence on pH (Table VII), although the possibility of a small secondary effect on final dry weight is not excluded.

Four strains of *Phycomyces blakesleeanus* were inoculated into ammonia medium without supplement. In 3 days the pH had fallen to 2·8 and by 7 days growth appeared to have ceased. An appropriate quantity of sodium bicarbonate or sodium succinate in solution was then added aseptically to some of the flasks, others being left as controls. The results are given in Table VII, whence it is clear that bicarbonate is as effective as succinate in causing renewed and extended growth. There does not seem to be any specific effect of succinate, although this acid is stated by Leonian and Lilly to be one of the most favourable to the growth of *Phycomyces*. Thus *Phycomyces* and *Mucor* fall in line with the fungi investigated earlier in their relation to organic acids, and do not exhibit requirements of a specific character. Our strains of these species were not, however, identical with those of Leonian and Lilly, and the existence of strains with such requirements is possible.

TABLE VII

Growth of Mucor ramannianus and Phycomyces blakesleeanus on ammonia medium with different supplements

Supplement	pH of medium		Dry matter (mg. per flask)	
Mucor	7 days	14 days	7 days	14 days
None	2·3	2·1	65	100
dl-malate	4·7	4·7	175	270
Succinate	4·8	4·9	155	275
Acetate	5·2	4·6	85	290
$CaCO_3$	6·5	5·3	190	—
KH_2PO_4	4·0	4·2	175	190
Phycomyces		19 days		19 days
None	2·8	2·7	32	36
Bicarbonate added at 7th day	6·2–6·9*	2·9–3·2	—	89
Succinate added at 7th day	5·3–5·7*	3·1–3·9	—	88

* pH immediately after addition of supplement.

Assimilation of nitrogen when different forms are present together

Estimations of nitrate and ammonia were made in culture experiments in which *Scopulariopsis* was grown on ammonium nitrate medium, both with and without addition of an organic acid salt (succinate). In the absence of succinate there is some utilization of ammonia (Table VIII), accompanied by a rapid fall in pH to the range 3·0–3·5, where growth is inhibited by hydrogen-ion concentration, after which nitrogen uptake ceases. Nitrate is not assimilated to any measurable extent.

When succinate is present the effect is to keep the pH steady at close to the initial value. The assimilation of ammonia is correspondingly more rapid than in the unbuffered medium, and here also there is practically no assimilation of nitrate for 5 days. Between the fifth and sixth day, however, nitrate assimilation begins quite suddenly and proceeds at approximately the same rate as ammonia assimilation and simultaneously with it, until all the nitrogen in the medium is utilized. The exact concentration of ammonia at which nitrate utilization begins cannot be determined in this experiment because samples were only taken at daily intervals. Nitrate utilization began at some time between the fifth and sixth day when the ammonia concentration was between 0·006 and 0·001 N.

These experiments have been repeated several times with identical results. They show that nitrate assimilation (even when the pH is favourable) is

TABLE VIII

Utilization of nitrate and ammonia from ammonium nitrate medium by Scopulariopsis brevicaulis

Days after inoculation	Nitrate in medium (mg. N per flask)	Ammonia in medium (mg. N per flask)	pH	Dry matter (mg. per flask)
		Succinate absent		
0	3·6	3·7	6·2	—
2	3·6	3·5	5·7	—
3	3·7	3·2	4·7	—
4	3·6	3·1	4·0	—
7	3·8	2·8	3·5	—
10	3·7	2·7	3·2	25
		Succinate present		
0	3·2	3·3	6·3	—
5	3·0	1·8	5·9	40
6	1·4	0·3	6·0	110
7	1·1	—	6·0	125
8	0·3	—	6·5	150
11	0·1	—	7·2	180

blocked in the presence of concentrations of ammonia nitrogen of the order commonly present in culture media, and they indicate that this blocking disappears when ammonia concentration falls below a certain level.

In order to define more closely the concentration of ammonia at which nitrate utilization begins, culture experiments were made with varying concentrations of ammonia in presence of the standard level of nitrate. The uptake of nitrate and ammonia was followed during the first few days. It was found that on no occasion did the fungus assimilate any measurable quantity of nitrate until the concentration of ammonia in the medium had fallen to 0·002 N. or lower.

As particular interest attaches to the behaviour of the fungus when both nitrate and ammonia are present in very low amounts, comparable to those in the soil solution, the fungus was grown in culture on a medium containing 0·0003 N. ammonia and 0·0009 N. nitrate. These concentrations are of the same order as those found in natural soils. Nitrate and ammonia determinations were made at 2-hour intervals. For 16 hours after inoculation there was no significant uptake of nitrogen; ammonia uptake then began and continued rapidly until at 22 hours it had all gone from the medium. There was no significant uptake of nitrate until all the ammonia had gone, then nitrate uptake began and continued rapidly. These results show that even at very low concentrations, such as occur in natural environments, ammonia completely suppresses the uptake of nitrate.

In the above experiments the mycelium developed from spores in a medium containing both nitrate and ammonia from the start. The evidence suggests that in these conditions the nitrate-assimilating system is fully developed but is blocked or inhibited until the ammonia in the medium has fallen to a low concentration, when it immediately begins to function with what appears to be maximal efficiency.

This interpretation is strengthened by the results of a culture experiment in which *Scopulariopsis* was grown from the beginning on nitrate medium. Ammonium sulphate was added to some flasks on the fourth day, whilst other flasks continued as controls without addition. The results show (Fig. 3) that mycelium receiving ammonia begins to assimilate it immediately, without lag, at a high rate, whilst the presence of ammonia completely stops nitrate assimilation within 24 hours of its addition. In the reverse experiment nitrate was added to 3-day cultures of the fungus growing on ammonia medium (with succinate present). Ammonia assimilation continued at the same rate as before and practically no nitrate was assimilated during at least 3 days whilst ammonia was still present (Fig. 4).

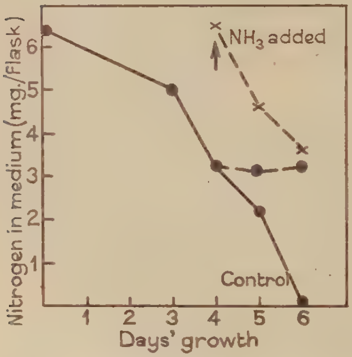

FIG. 3.

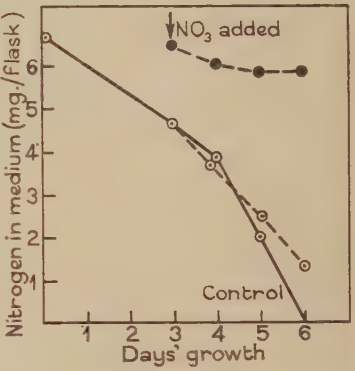

FIG. 4.

FIG. 3. *S. brevicaulis;* effect of addition of ammonia on the uptake of nitrate from the medium. Culture in nitrate only (——); culture in nitrate and ammonia (– – – –); ● nitrate-N; × ammonia-N.

FIG. 4. *S. brevicaulis;* effect of addition of nitrate on uptake of ammonia from the medium. Culture in ammonia (——); culture in ammonia and nitrate (– – – –); ● nitrate-N; ⊙ ammonia-N.

In a further experiment the fungus was grown in shake culture in nitrate medium and in ammonia medium (with succinate). Actively growing mycelium from each substrate was then transferred, after removal of the medium by suction filtration, but without washing, to nitrate, ammonium sulphate, and ammonium nitrate, in glucose+buffer. Uptake of different forms of nitrogen was measured over a period of 6 hours. From the results (Table IX) it may be seen, first, that nitrate uptake is reduced in the presence of ammonia, although not completely stopped in these conditions, and secondly, that mycelium previously grown on ammonia develops the enzyme systems concerned in nitrate reduction and assimilation, and can assimilate nitrate

TABLE IX

Uptake of different forms of nitrogen by Scopulariopsis brevicaulis previously grown on either nitrate or ammonia (shake culture)

Previous nitrogen-substrate	Nitrate			Ammonia		
Transferred to . .	NH_3	NO_3	NH_3+NO_3	NH_3	NO_3	NH_3+NO_3
Uptake of nitrogen (mg. N/g. dry matter in 6 hours) . .	12·5	8·8	5·9+4·0	10·3	5·4	6·2+2·4

immediately at a rate comparable with, although somewhat lower than, nitrate-grown mycelium. Thus the nitrate-reducing system is formed in the complete absence of nitrate but is inactive, and only begins to function when ammonia is removed.

It is evident from these facts that nitrate assimilation is normally prevented in *Scopulariopsis* by the presence of ammonia at more than a low critical concentration. Before it can be built into protein and other cell substances nitrate must be reduced, either to ammonia itself or at least to some compound close to ammonia on the path of protein synthesis. Since additional energy is required for the reduction of nitrate to this level, and since further its assimilation seems to be slower than that of ammonia, the latter might be expected to be used preferentially. From this point of view the blocking of nitrate assimilation by ammonia would represent a type of mass-action effect, the replacement in metabolism of one nitrogen substrate by another more readily assimilated.

The results could, alternatively, be explained if ammonia acted as a specific inhibitor, blocking some stage in nitrate reduction. Some evidence for the latter view comes from a study of nitrite assimilation.

Scopulariopsis grows quite normally on standard culture medium in which nitrogen is present as potassium nitrite, and in course of growth all the nitrite is taken up. It may be noted that in nitrite culture medium the concentration of nitrite is equivalent to that of nitrate or ammonia in the corresponding media, and is far higher than ever occurs in the medium when the fungus is growing on nitrate, when, as an intermediate, traces of nitrite are sometimes present. The fungus will also grow satisfactorily on ammonium nitrite medium (made up by mixing ammonium sulphate and potassium nitrite, and sterilized without heat by Seitz filtration). Its behaviour on ammonium nitrite is quite distinct from that on ammonium nitrate. There is no fall in pH during growth, even when no organic acid salt is added, indicating that nitrite and ammonia are taken up together. Estimations of nitrite and ammonia in the medium show that both nitrogen sources are utilized at approximately the same rate (Table X).

The same general features are shown in another culture experiment, where the fungus was grown on varying proportions of nitrite and ammonia (lower half of Table X), fumarate being present in all media to obviate pH changes.

In each case nitrite and ammonia are taken up simultaneously and at comparable rates. Ammonia is absorbed slightly more rapidly than nitrite, and this is most marked in the medium with the highest concentration of nitrite relative to ammonia.

The same relation between nitrite and ammonia uptake is also found when ammonia is added to a rapidly growing culture of the fungus on nitrite medium. Ammonium sulphate was added when the mycelium, growing on nitrite, was 5 days old. The subsequent behaviour is illustrated in Fig. 5, which shows that ammonia uptake begins immediately after its addition: nitrite uptake continues, however, in the presence of ammonia at only a slightly reduced rate.

TABLE X

Uptake of nitrogen by Scopulariopsis brevicaulis from ammonium nitrite medium

	Without fumarate			With fumarate		
	Nitrogen present (mg. N per flask)		pH	Nitrogen present (mg. N per flask)		pH
Time (days)	Ammonia	Nitrite		Ammonia	Nitrite	
Initial	3·4	2·9	6·4	3·4	2·9	6·4
2	3·0	2·6	6·1	2·7	3·0	6·3
3	2·7	2·5	5·9	2·0	1·8	5·9
4	1·2	1·3	5·5	0·9	0·9	5·7
Dry matter at 4 days (mg./flask)	36			22		

	With fumarate Nitrogen present per flask (mg.)					
Time (days)	Ammonia	Nitrite	Ammonia	Nitrite	Ammonia	Nitrite
Initial	1·6	4·1	3·2	2·8	4·5	1·4
3	1·0	4·0	2·6	2·3	3·4	0·8
4	0·4	3·2	1·6	1·5	1·6	0·3
5	—	1·2	—	0·4	—	—
7	—	—	—	—	—	—
Dry matter at 7 days (mg./flask)	82		80		78	

The reverse experiment, the addition of nitrite to a culture growing on ammonia medium, gives different results. It appears that nitrite in the concentration used (approximately equivalent to ammonia in standard medium) is somewhat toxic to the mycelium, unless the fungus has acquired tolerance by previous growth on relatively high concentration of nitrite. Thus the effect of adding nitrite to cultures of the organism on ammonia is to stop the assimilation of both forms of nitrogen for 1–2 days. After this interval the fungus begins to assimilate both nitrite and ammonia simultaneously.

That nitrate and nitrite are assimilated concurrently is evident, since nitrite does not accumulate appreciably when the fungus is growing on nitrate. Culture experiments in which nitrate and nitrite are supplied together at the same concentration show that the level of each is reduced at almost the same rate during growth. This implies that nitrite is actually being used more rapidly than nitrate in these conditions, which is not unexpected in view of the unusually high concentration of nitrite relative to nitrate, and of the fact that the mycelium is adapted by growth on high concentration of nitrite from the start. When nitrite, at a concentration equal to the nitrate present and therefore much higher than the trace normally occurring, is added to mycelium growing on nitrate medium, there is some evidence that nitrate assimilation is partially or wholly blocked, although assimilation of the nitrite proceeds rapidly.

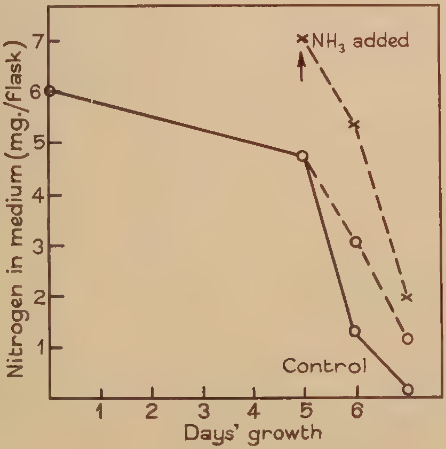

FIG. 5. *S. brevicaulis;* effect of addition of ammonia on the uptake of nitrite from the medium. ○ nitrite-N; × ammonia-N; (——) culture in nitrite only; (– – – –) culture in nitrite and ammonia.

These temporary inhibitory or toxic effects of nitrite have not been fully investigated. They are only apparent when the fungus is suddenly subjected to an unusually high concentration of nitrite. In culture the fungus rapidly becomes tolerant of concentrations of nitrite equivalent to the nitrate or ammonia in standard media, and is able to assimilate the nitrite completely and grow normally.

Blocking of nitrate assimilation by ammonia

The important fact which emerges from these experiments is that nitrite-adapted mycelium assimilates nitrite simultaneously with ammonia, although somewhat more slowly than when ammonia is absent. Since, on the contrary, ammonia inhibits nitrate assimilation, the effect of ammonia must be exercised on the first stage of nitrate reduction, prior to nitrite. In other words, ammonia specifically interferes with the reduction of nitrate to nitrite.

The suppression of nitrate assimilation by ammonia appears to be a general phenomenon in fungi, not confined to *Scopulariopsis*. When cultured on ammonium nitrate the following species have been shown not to utilize nitrate at all in the early stages of growth and to begin to take up nitrate only when the ammonia has fallen to a low level: *Alternaria solani*, *Aspergillus repens*, *Botrytis allii*, *Cladosporium herbarum*, *Diplodia natalensis*, *Mucor ramannianus*, *Myrothecium verrucaria*, *Penicillium chrysogenum*. When nitrate-grown mycelium of *Aspergillus nidulans*, *Botrytis allii*, *Penicillium chrysogenum*, and *Trichoderma viride* is transferred (in replicate) to nitrate, ammonium nitrate, and ammonia medium, the rate of nitrate uptake is reduced by 42 to 82 per cent. in the presence of ammonia, whilst the rate of ammonia uptake is only reduced by 6 to 11 per cent. in the presence of nitrate in the first 5 hours after transfer.

There are, however, some species, e.g. *Aspergillus niger*, *A. oryzae*, *Fusarium graminearum*, *Penicillium griseofulvum*, in which nitrate appears to be used at the same time as ammonia, although relatively more slowly.

DISCUSSION

The unusual pattern of nitrogen nutrition in *Scopulariopsis brevicaulis*, *Myrothecium verrucaria*, and some other fungi, is explained by the experiments which have been reported, and seems to be due to the great sensitivity of these fungi to the adverse effects of low pH.

In common with many others these fungi do not assimilate nitrate when ammonia is present. For this reason they behave on ammonium nitrate in the same way as on ammonium sulphate, in spite of their ability to assimilate nitrate. The effect of organic acids on ammonia assimilation has been shown to be primarily a buffering effect on the pH drift of the culture medium. This finding is not in agreement with that of Brian, Curtis, and Hemming (1947), who concluded that *Myrothecium verrucaria* and *Scopulariopsis brevicaulis* show a specific requirement for organic acids when assimilating ammonia nitrogen.

The two main lines of evidence presented by Brian, Curtis, and Hemming against a buffering effect were as follows. Firstly, with small supplements of acids (0·1 per cent.) the pH drift with *M. verrucaria* was with several acids the same as on ammonia medium without supplement but the growth produced was much greater. Actually, however, the pH drifts were the same only after the first week, the fall in pH on the supplemented media being distinctly slower for some days after inoculation, while the dry weights were determined only after 2 or 3 weeks. In the present investigation experiments made with weak buffers make it probable that a higher pH of the medium during the first few days (less than four) is sufficient to produce an increase in growth.

In the second place, these authors found that in ammonia medium the increase in growth due to a higher initial pH of the medium was small compared with the increase in growth produced by an organic acid supplement

(1 per cent. malic). However, their figures show that in media without acid supplement adjusted initially to pH 4, pH 5, and pH 6·5, the pH dropped to 3 or lower by the 5th, 11th, or 15th day respectively. In the presence of the acid supplement, on the other hand, the pH never fell below these initial values.

These results are not, therefore, at variance with the more detailed experiments with *S. brevicaulis* and *M. verrucaria* reported in this paper. It must be remembered in this connexion that, whilst the primary effects of organic acids have been shown to be on pH drift and only through this on ammonia assimilation and growth, certain secondary effects on growth have also been observed depending both on the nature of the organic acid added and the particular fungal species. There is, however, no evidence that fungi, supplied with glucose, require any exogenous supply of organic acid for the assimilation of ammonia provided the reaction of the medium is favourable. Many instances of the effect of organic acids in improving the growth of fungi are reported in the literature, but in all the species that have been examined in the present investigation this effect has been found to be due principally to the buffering action of the acids. Moreover, where pH data are given, the evidence reported in the literature is consistent with this conclusion.

The supplying of acetate, pyruvate, or several acids of the Krebs cycle to the growing fungus has no effect on the rate of nitrogen uptake when glucose is present, whilst nitrogen uptake ceases immediately if glucose is removed and replaced by one of these acids. This is a fact of some significance if these acids, either directly or formed through transformations of the Krebs cycle and related pathways, are considered to be a source of the carbon skeletons required for the building up of amino-acids and proteins. It would seem to imply that the internal concentration of essential organic acids is maintained at levels which do not limit nitrogen uptake as long as glucose is being metabolized and are unaffected by exogenous supply of intermediary metabolites.

The suppression of nitrate assimilation by ammonia at very low concentration appears to be characteristic of very many fungi which can assimilate both these forms of nitrogen, and since nitrite uptake is almost unaffected, it is likely that ammonia inhibits in some way the reduction of nitrate to nitrite.

The general occurrence of ammonia-induced inhibition of nitrate uptake in fungi suggests an adaptive significance. In natural habitats, such as the soil, ammonia and nitrate frequently occur together. Nitrate requires a greater expenditure of energy for its assimilation than does ammonia; it is probably to the advantage of the organism, therefore, to assimilate ammonia first, since the supply of energy available in the form of suitable carbon substrates may be the most important factor limiting growth in such habitats (see Wright, 1953).

It is noteworthy in this connexion that ammonia virtually excludes nitrate uptake when the two are present at low concentration of the order found in the soil water in field conditions. Thus the ammonia-nitrate antagonism is significant in a natural environment and not only in artificial media. Nitrite, on the other hand, although it is assimilated concurrently with ammonia, is not usually present in more than very minute amounts in natural environments.

In conditions of pH favourable for growth the rate of uptake of ammonia is more rapid than that of nitrate. Results of experiments involving transfer of mycelium from one nitrogen source to another show that the nitrate-reducing system is developed, either fully or to a considerable extent, in the complete absence of nitrate from the culture medium. This is the condition in certain nitrate-reducing bacteria except that here the nitrate-reducing system is more markedly adaptive (Pollock, 1946).

Nitrate-grown mycelium, on the other hand, is fully adapted to assimilate ammonia. These observations suggest that the assimilation of nitrate and ammonia proceed to a considerable extent over the same path. It seems probable, however, that nitrate does not actually pass through ammonia, in view of the fact that the latter inhibits nitrate reduction, but rather that both nitrate and ammonia enter metabolism through a common stage.

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The Amino-Acids of Grass Pollen

N. O. BATHURST

Grasslands Division, Palmerston North, New Zealand

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SUMMARY

Pollens collected from nine grass species were analysed for ammonia, urea, glutamine, asparagine, amino-nitrogen, total soluble nitrogen, and total nitrogen. Four grass pollens were also assayed microbiologically for 17 individual amino-acids.

Wide variations in composition were found between pollens from different grasses. Soluble nitrogen was in most cases relatively high in proportion to the total nitrogen. Asparagine occurred in much greater amount than glutamine. Of the free amino-acids, the most abundant was proline, its content in the perennial ryegrass pollen reaching 1·65 per cent. of the dry weight of the pollen

INTRODUCTION

THE chemical composition of pollen is of great interest, not only because of the important part pollen plays in the reproductive process, but also because of the influence of many pollens, particularly grass pollens, on the incidence of hay fever. Particular attention has been paid to the skin-reactive fraction, which has been found to be largely protein or protein-carbohydrate (Robbins, Samuels, and Mosco, 1948).

Detailed analysis of the nitrogenous constituents of pollen include those of Ray Sarker *et al.* (1949), who measured by microbiological assay the concentration of 12 amino-acids in hydrolysed sweet corn pollen. The amino-acids estimated would therefore include those present in the free state as well as those present in bound forms including peptides and proteins. A qualitative analysis of amino-acids was performed by Auclair and Jamieson (1948), using two-way paper chromatography. They prepared alcohol extracts and also hydrolysates of the alcohol-insoluble residue of dandelion, willow, and mixed pollen, all the pollens being collected by bees. They found up to 22 amino-acids, with considerable variation between species. β-alanine and α-aminobutyric acid were sometimes present.

The purpose of the present investigation was to obtain quantitative information on the soluble amino-acids, both free and bound, of some grass pollens.

EXPERIMENTAL

The grasses selected were transferred when almost mature to trays in the glasshouse, so that the plants were inclined with their tops over sheets of paper. As the pollen was shed (assisted by shaking) it fell on to the paper and was allowed to remain there until air-dry. Anthers mixed with the pollen were removed by sieving. The four samples used for microbiological assay of

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amino-acids were dried in a vacuum desiccator over calcium chloride instead of being air-dried.

Extracts used for estimation of ammonia, urea, amides, amino-nitrogen, and total soluble nitrogen were prepared by extracting 2 g. pollen with water containing 0·3 ml. acetic acid in the Waring Blendor, making up the extract with water to 100 ml., and centrifuging off the residue.

For the assay of individual amino-acids, samples of 17 to 20 g. were extracted with four lots each of about 500 ml. of 80 per cent. ethyl alcohol in the Waring Blendor. The combined alcohol extracts were concentrated under reduced pressure until all the alcohol was removed, the residue being made up with water so that 1 ml. of extract was equivalent to 500 mg. pollen. The pollen residue insoluble in alcohol was extracted with water in the blendor and the extract hydrolysed and analysed by conventional methods.

Table I shows the amounts of ammonia, urea, glutamine, asparagine, amino-nitrogen, total soluble nitrogen, and total nitrogen for the pollen of 9 species of grasses. These samples had been air-dried and stored for 1 year before analysis.

TABLE I

Nitrogenous fractions of grass pollens expressed as mg N per cent.

Source	NH_3-N	Urea-N	Glutamine amide-N	Asparagine amide-N	Amino-N	Total sol. N	Total N
Italian rye . .	4·3	3·4	19·9	38·0	202	947	3030
Chewings fescue .	7·6	0·2	11·8	17·6	154	753	2950
Crested dogstail .	3·7	0·5	18·4	80·4	400	1,305	2900
Timothy . .	15·4	1·7	39·0	112·9	326	1,210	3980
Perennial rye . .	4·6	6·5	12·6	42·1	178	930	2820
Cocksfoot . .	5·4	0·5	8·1	34·2	167	515	2720
Yorkshire fog . .	7·2	1·0	13·1	54·1	203	815	3500
Tall fescue . .	19·2	0	21·7	49·5	235	1,095	3980
Meadow foxtail .	13·3	0	1·7	27·0	105	729	3270

Results for the microbiological assay of 17 amino-acids, both free and bound, in the soluble fractions of four different grass pollens are shown in Table II. These samples were analysed shortly after being collected and dried in a vacuum desiccator.

All results are expressed as per cent. or mg. per cent. of the fully dried material.

It is apparent that there is considerable variation in composition between the different species of pollen. The proportion of soluble to total nitrogen is higher than normally found in leaf tissue, varying (for the samples included in Table I) from 19 to 45 per cent. The content of soluble nitrogen shown for these samples, however, may have been affected by the storage of the pollen for 1 year at a moisture content of about 5 per cent. For the fresh samples, as shown in Table II, the total soluble nitrogen (taking the sum of the nitrogen in the alcohol and water extracts) is from 15 to 18 per cent. of the total nitrogen.

TABLE II

Amino-acids in grass pollen in mg. amino-N per 100 g. dry matter

	Chewings fescue		Cocksfoot		Perennial rye		Timothy	
	Alc.	Water	Alc.	Water	Alc.	Water	Alc.	Water
Alanine	16·3	15·4	30·8	13·2	15·7	11·6	17·0	21·4
Arginine	0·3	1·9	1·0	2·0	1·3	1·4	0·3	3·1
Aspartic acid + asparagine	25·2	9·7	46·2	8·4	17·2	8·6	36·1	11·3
Glutamic acid + glutamine	7·4	5·3	10·8	—	8·9	7·7	16·3	10·6
Glycine	10·9	7·9	12·4	7·5	18·7	7·5	6·7	10·9
Histidine	2·0	1·2	2·3	1·2	3·1	1·4	3·1	2·7
Isoleucine	5·8	3·3	6·5	3·3	4·3	2·9	4·3	3·9
Leucine	2·6	2·8	3·4	3·0	3·5	4·0	2·8	6·9
Lysine	2·3	8·6	2·5	6·7	3·4	8·5	2·1	10·9
Methionine	2·1	2·5	2·3	2·6	0·5	1·0	0·9	1·2
Phenylalanine	1·5	1·5	1·7	2·5	1·8	2·5	2·0	3·1
Proline	146·5	6·8	40·2	4·4	201·5	4·6	97·7	7·3
Serine	8·8	4·4	32·6	4·0	9·9	4·7	10·1	6·0
Threonine	3·1	3·3	5·1	3·2	5·8	3·8	6·3	5·9
Tryptophane	0·3	—	0·4	—	0·3	—	0·9	—
Tyrosine	1·4	1·2	2·2	1·0	2·2	1·3	2·8	1·8
Valine	6·5	3·6	5·6	3·6	8·0	4·4	6·0	6·8
Total (by summation)	243·2	79·4	166·0	—	306·1	75·9	205·4	113·8
Total (Van Slyke)	92	85	107	92	83	91	178	112
Total soluble N (mg.%)	464	109	464	93	490	117	424	159
Total nitrogen (%)	3·44		3·11		3·42		3·81	

The figures for individual amino-acids (Table II) show the same wide variation as do the figures in Table I. One striking feature is the large amount of proline imino-nitrogen which, in the perennial ryegrass pollen, is equivalent to a content of 1·65 per cent. of free proline in the dry pollen. Of the other amino-acids, the most abundant are the dicarboxylic acids with their amides, alanine, glycine, serine, and valine. This is in line with what is normally encountered in leaf tissue.

In the alcohol extracts, the anomalous results for total amino-nitrogen by Van Slyke's nitrous acid method compared with the corresponding figure obtained by summation of the amino-nitrogen of the individual acids would be largely due to the fact that proline nitrogen is not included in that measured by Van Slyke's method. If the proline-nitrogen figures are excluded from the summation a much closer agreement is obtained.

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Soluble Nitrogenous Constituents of Lupin Nodules

N. O. BATHURST

Grasslands Division, Palmerston North, New Zealand

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SUMMARY

Lupin nodules collected in two successive years were analysed for ammonia, urea, glutamine, asparagine, amino-nitrogen, peptide-nitrogen, total soluble nitrogen, and total nitrogen at fortnightly or weekly intervals from the time the plants reached full flower. The amounts of many of the constituents passed through a maximum, in the first experiment about a month after flowering, in the second at or a few days after flowering.

On a sample taken a week after full flowering, assays were made of 17 individual amino-acids in three fractions representing free amino-acids, peptides, and soluble protein. Of the free amino-acids, those present in the greatest amount were aspartic and glutamic acids together with their amides, alanine, serine, histidine, and valine. The other two extracts showed a more even distribution of amino-nitrogen among the different amino-acids.

INTRODUCTION

As most nodules are very small, the collection of a sufficient amount is very tedious. For this reason much previous work on the nitrogenous constituents of nodules has been qualitative or semi-quantitative. Hunt (1949, 1951) used paper chromatography for a qualitative analysis of the free amino-acids of the nodules and roots of garden bean, garden pea, soybean, red clover, and lucerne. He showed the presence in these nodules of usually at least 12 known amino-acids (including γ-aminobutyric acid and β-alanine) with up to 3 unknown ninhydrin-reacting substances. Virtanen and Miettinen (1952), using a semiquantitative paper chromatographic method, found that the most abundant amino-acid in the roots and nodules of the alder was citrulline, which sometimes amounted to 1 to 2 per cent. of the dry weight of the nodules. Zelitch, Wilson, and Burris (1952) measured the amino-acid composition of soybean nodules by partition chromatography on starch columns. They found the most abundant amino-acids to be arginine, glutamic acid, lysine, and aspartic acid.

EXPERIMENTAL

Material. Blue lupins were used, as their nodules are large and comparatively easy to separate; often as much as 5 g. of nodules can be obtained from a single plant.

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Sampling and extraction. Samples were taken from the time the plants were in full flower, as before this time the nodules were so small that collection was difficult. In the first experiment the first sample was taken on 8 February 1950, successive samples being taken at fortnightly intervals. In the second experiment sampling began on 15 January 1951, when 2 samples were taken, the first consisting of plants still in flower and the second of plants that had formed pods. Thereafter samples were taken weekly. In each experiment 7 sets of samples were taken. The number of plants taken for each sample varied from 30 to 120, depending on the size of the nodules.

As soon as the plants were dug the roots were removed, washed with water, and the nodules detached and weighed. Samples of 20 g. were extracted in the Waring Blendor according to the method of Reifer and Melville (1951).

The sample used for analysis of individual amino-acids was taken on 15 February 1950, the nodules removed and freeze-dried. 50 g. of freeze-dried nodules were thoroughly extracted in the Waring Blendor with 80 per cent. ethyl alcohol. The residue was extracted with water and centrifuged. The protein was precipitated from the aqueous extract with acetic acid and was hydrolysed by refluxing with 6 N. HCl for 16 hours. This separation is more fully described in a previous paper (Bathurst, 1953).

Analytical methods. Conventional methods were used for most fractions. Ammonia was estimated by the hypobromite method of Reifer (1938) using the distillation apparatus of Pucher, Vickery, and Leavenworth (1935).

Individual amino-acids were estimated by microbiological assay, the organisms used being *Leuconostoc mesenteroides* (for histidine, lysine, phenylalanine, methionine, threonine, aspartic acid, tyrosine, proline, and glycine); *Lactobacillus arabinosus* (for tryptophane, leucine, isoleucine, valine, and glutamic acid); *Streptococcus faecalis* (for arginine); and *Leuconostoc citrovorum* (for alanine). The assays were performed in 4 × ⅝-inch test-tubes, the total volume of each tube being made to 1 ml. The response was measured by titration of the acid produced, with 0·05 N. NaOH, the maximum titration usually reaching 2–3 ml. Supplements for the alanine and glutamic acid media were prepared by paper chromatography as described by Bathurst (1953).

RESULTS

Table I shows the results of the two experiments. The sample of nodules for microbiological assay of amino-acids was taken 1 week after the first sample of the first experiment. Results are shown in Table II.

The data show that the nodules contained a high proportion of soluble to total nitrogen (31 to 38 per cent.). Total nitrogen was at all samplings higher in the nodules than in any other part of the plant. It is apparent, too, that in most cases the amounts of the different constituents and groups measured pass through definite maxima at the third or fourth sampling, i.e. at 4 to 5 weeks after flowering. The urea nitrogen is comparatively high, sometimes

TABLE I

Nitrogenous fractions of lupin nodules expressed as mg. N per cent. (N.D. = no determination)

Sample no.	Date	NH_3-N	Urea-N	Glutamine amide-N	Asparagine amide-N	Total sol. N	Amino-N	Peptide-N	Rest-N	Total N	Insol. N
	1950										
1	6 Feb.	22·2	5·5	42·6	80	1,710	464	N.D.	—	4,890	3,180
2	21 Feb.	14·5	14·9	30·6	138	1,530	446	N.D.	—	4,920	3,390
3	7 Mar.	24·0	22·3	38·9	236	1,900	531	N.D.	—	5,520	3,620
4	20 Mar.	17·1	22·3	30·9	171	1,808	522	358	687	5,200	3,390
5	3 Apr.	30·6	15·5	19·1	167	1,860	453	545	630	5,400	3,540
6	17 Apr.	24·3	9·0	23·8	146	1,780	392	461	724	4,790	3,010
7	1 May	24·6	14·4	17·5	158	1,562	426	220	702	4,260	2,700
	1951										
1	15 Jan.	29·6	7·4	39·3	374	1,845	634	386	375	5,230	3,390
2	15 Jan.	26·2	10·8	32·7	381	2,160	735	432	542	4,980	2,820
3	23 Jan.	30·9	9·0	19·2	256	1,490	506	292	377	4,380	2,890
4	29 Jan.	17·9	17·7	14·4	166	938	254	260	208	3,790	2,850
5	5 Feb.	34·3	13·1	18·6	111	920	254	285	204	3,500	2,580
6	12 Feb.	24·3	30·1	6·7	131	841	205	273	171	3,280	2,440
7	19 Feb.	38·3	14·0	7·4	101	725	211	240	113	3,400	2,680

TABLE II

Amino-acids in lupin nodules (expressed in mg. amino-N per 100 g. dry matter)

	Alcohol extract	Water extract hydrolysed	Acetic acid ppt. hydrolysed
Alanine	16·5	10·1	6·6
Arginine	1·3	3·5	11·3
Aspartic acid + asparagine	134	21·3	14·5
Glutamic acid + glutamine	36·5	8·6	12·0
Glycine	3·2	9·5	10·7
Histidine	5·8	1·8	2·0
Isoleucine	3·9	3·5	7·7
Leucine	2·2	8·3	2·8
Lysine	0·5	7·6	6·1
Methionine	0·4	3·4	1·1
Phenylalanine	1·7	1·7	2·0
Proline	2·6	4·5	6·6
Serine	9·6	3·9	5·9
Threonine	3·0	2·8	3·1
Tryptophane	2·7	—	—
Tyrosine	1·2	1·5	3·3
Valine	5·2	2·9	6·9
Total (by summation)	230·3	89·4	108·1
Total (Van Slyke)	359	110	152
Recovery per cent.	64	81	71

exceeding that due to ammonia or glutamine. The figures for ammonia and urea show less regularity than do the others. A noteworthy feature is the much greater amount of asparagine than glutamine. Estimations of peptide nitrogen were not commenced until the fourth sampling, but even when these figures are taken into account there still remains a large proportion (34 to 45 per cent.) of the total soluble nitrogen that is unidentified (Rest-Nitrogen).

The second experiment, performed nearly a year later, in general confirmed the results of the first experiment. The maxima in the various groups, however, occurred much earlier, at or a few days after flowering. Asparagine, total soluble nitrogen, and amino-nitrogen all attained higher maxima and lower minima than in the first experiment.

A total of 17 amino-acids were assayed on the nodule sample taken for this purpose. Asparagine and aspartic acid were measured together (after hydrolysis) as the assay of aspartic acid in the presence of much asparagine is unreliable (Bathurst, 1953). Glutamine and glutamic acid were also measured together. In the two acid-hydrolysed extracts, of course, these two amides are completely converted to their acids. Tryptophane is destroyed by acid hydrolysis and so is not found in the acid-hydrolysed extracts.

In the alcohol extract containing the free amino-acids, the dicarboxylic acids with their amides account for over 70 per cent. of the amino-nitrogen as determined by summation. Although glutamine and asparagine were not determined on the sample used for amino-acid analyses it would appear from samples 1 and 2 in 1950 that the dicarboxylic acids are present largely as heir amides. Alanine, serine, histidine, and valine are next in order of mportance, but of these only histidine deserves mention. In other plant issues examined in this laboratory free histidine has rarely been found in nore than traces. The amount of free tryptophane, too, greatly exceeds that ormally found in leaf tissue, although higher amounts have been found in oots of lupin (7·1 compared with 2·7 mg. per cent. in nodules). Levels of ree arginine and lysine were not markedly different from those in other plant issues examined. This is of interest when compared with the results of Zelitch *et al.* (1952), who found high levels of both these amino-acids in a ydrolysate of whole soybean nodules.

The hydrolysed water extract shows a more even distribution of amino-itrogen among the different amino-acids, which are believed to be derived mainly from peptides (Bathurst, 1953). The value for methionine in this xtract is markedly higher than normally found for leaf tissue.

The acetic acid precipitate representing at least 7 per cent. of the total oluble nitrogen is a fraction hitherto encountered only in traces in plant issues in this laboratory. It is believed to be a protein or mixture of proteins vhich after freeze-drying and treatment with cold 80 per cent. alcohol is still oluble in water at hydrogen-ion concentrations above the isoelectric point.

The quoted analyses bring into sharp relief the discrepancies which exist in current methods of nitrogen partition in plant tissues. The amount of amino-nitrogen calculated from the sum of the assays of individual amino-acids in every case fell short of the amount indicated by the Van Slyke nitrous acid method, but the ratios of the two figures were similar to those found for other tissues (Bathurst, 1953). This discrepancy could result, not only from the inclusion of non-amino nitrogen in the Van Slyke measurement, but also from the omission of amino-acids such as γ-aminobutyric acid from assay.

Analyses of whole nodules, as described in this and in other papers, clearly indicate the difference in composition between these structures and other plant organs. It must be stressed, however, that the whole nodule is not a homogeneous tissue, since the bacterial mass, in which the actual process of nitrogen fixation is presumably centred, is surrounded by an envelope of metamorphosed root tissue which plays no part in the process. Hence any compound which is peculiar to, or which exists in high concentration in the actively fixing tissue, will be diluted owing to the enveloping root tissue. The dissection of nodules into these two components would undoubtedly demonstrate differences in the nitrogen fractions which are not brought out in the analyses of whole nodules.

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The Relation between Concentration of Hydrogen Ions and the Effect of Weak Acids or Bases on Metabolic Activity

G. E. BRIGGS

Botany School, Cambridge

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SUMMARY

The relation between the concentration of hydrogen ions and the effect of a weak acid on metabolic activity as indicative of the role of ions and molecules is considered. Special reference is made to a metabolic system containing non-mobile anions. The consequence of substituting divalent cations for univalent is indicated.

IN reports of investigations of the effect of change of pH of the external medium on the activity of cells or tissues in solutions of weak acids or bases conclusions have been drawn about the form, ion or molecule, in which the electrolyte penetrates the cell and about the relative effect of the ions and molecules of the electrolyte on the metabolic activity. It is the purpose of this paper to indicate some of the principles which must be borne in mind in drawing such conclusions.

There is little to be said on the topic of form of penetrant. Since the rate at which an acid or base comes into equilibrium with its ions is very rapid, we can assume that the equilibrium between molecules and ions is not disturbed by the rate of arrival or rate of removal or by the form in which the substance enters or is removed. The time required for the concentration inside to come to equilibrium with that outside will be greater if the form in which it penetrates is a small fraction of the total. Hence the form in which the substance penetrates will determine the rate at which a metabolic effect will develop, but it will not determine the magnitude of the effect finally developed.

The concentration (or more precisely the activity) of the ion at any point can be deduced from the pH and the concentration of the molecules. If the substance is not being consumed at, or actively transported from, any point in the tissue, then, when equilibrium is reached, the concentration of the molecules of the substance will necessarily be the same at all points as in the external solution. If the substance is being consumed in the cell cytoplasm, for example, or actively transferred from the cytoplasm to the vacuole at a constant rate, then a steady state will be reached when the concentration in the cytoplasm is some fraction of that outside—a fraction dependent upon such factors as coefficient of diffusion, length of diffusion path from outside

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to the cytoplasm which will differ from cell to cell in a piece of tissue, velocity constant of removal from cytoplasm, &c. If the substance is being removed in this way, then the concentration in the outside medium will fall with time.[1]

Let us consider a tissue in which the substance which affects the metabolic activity is not being removed in any way by the tissue, and to be specific let the substance be a weak acid, HA, which depresses some metabolic activity. The weak acid may exert its depressant effect by combining either with a catalyst, competing or not competing with a substrate, or by combining with a substrate.

There are various possibilities including (*a*) molecules of HA and not ions can combine with X, a metabolic catalyst or substrate, giving an ineffective complex; (*b*) both ions, A^-, and molecules can combine but only XHA is ineffective; (*c*) both combine and both XA^- and XHA are ineffective.

We could try to distinguish between these three by changing the relative concentrations of HA and A^- around X. If HA were maintained constant while A^- increased with increasing pH, constancy of depressant effect would indicate the first possibility. In the second case the depressant effect should decrease because A^- ions would compete with HA molecules for X, whilst in the third case the depressant effect should increase because the total XHA plus XA^- would increase except when HA is enough to saturate X.

If, however, the total of molecules, HA, plus ions, A^-, is maintained constant while the latter is increased, then further information may be required. Let us be more specific and make the following assumptions; that HA combines reversibly with X with a dissociation constant k_A; and that for cases (*b*) and (*c*) the acidic dissociation constant of HA, namely k_a, is not affected by combination with X, in other words that the affinity of X is the same for A^- as for HA; and further that HA competes with nothing else for X. Then if the total of all forms of X is x

$$[\mathrm{XHA}] = \frac{x[\mathrm{HA}]}{[\mathrm{HA}]+[\mathrm{A}^-]+k_A}$$

$$\text{and } [\mathrm{XA}^-]+[\mathrm{XHA}] = \frac{x([\mathrm{HA}]+[\mathrm{A}^-])}{[\mathrm{HA}]+[\mathrm{A}^-]+k_A}.$$

The expression for case (*a*) is

$$[\mathrm{XHA}] = \frac{x[\mathrm{HA}]}{[\mathrm{HA}]+k_A}.$$

Commencing with a pH so low that practically all the depressant is in the form HA, let us consider the consequences of increasing the fraction ionized by increasing the pH. Proportionality of depressant effect to [HA] is consistent with case (*a*) only as long as $[\mathrm{HA}] \ll k_A$ and the metabolic activity of X is proportional to that which is free of depressant. When [HA] is not negligible compared with k_A and if the activity of X rises less rapidly than the concentration of free X, as it may do if X is a metabolite rather than a catalyst, then the depressant effect would fall less rapidly than the concentration of HA. Hence such a consequence cannot be taken as indicating a depressant effect of the ion A^-.

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[1] Experiments in this laboratory have shown that carrot disks in 10 times their volume of 0·00125 M. sodium malonate, a respiratory inhibitor, reduce the external concentration to a half in 10 hours. The fall would be more rapid if the ratio of liquid to tissue were smaller.

For case (*b*) the depressant effect will fall in proportion to the fall in [HA] whatever its value relative to k_A provided the activity of X is proportional to that which is free. Again, if the activity of X rises less rapidly than free X, the depressant effect will fall less rapidly than HA does. For case (*c*) there are no complications since $HA+A^-$ remains constant, the amount of X free from depressant and hence the depressant effect remains unchanged.

For isolated catalytic systems we may be able to fix both HA and A^- at known concentrations, but with the catalytic systems inside the cytoplasm we can fix [HA], since it is the same as in the outside solution if there is no traffic in HA, but $[A^-]$ will be the same inside as out only if the pH is the same in both.

There is evidence that the pH in the cytoplasm of plant cells is generally lower than that in the external solution. Let us assume that this is due to a non-diffusible protein existing in the form of molecules HP and anions P, the relative amounts depending upon the dissociation constant k_p and the pH. For such a tissue the concentration of diffusible cations will be greater inside than out and of anions less as long as these are non-diffusible anions. When the diffusible cations are all univalent the following relations hold:

$$\frac{K_i}{K_0} = \frac{[H_i^+]}{[H_0^+]} = \frac{[P]+[A_i^-]+[OH_i^-]}{[A_0^-]\,[OH_0\,]} = \frac{[A_0^-]}{[A_i^-]} = \frac{[OH_0^-]}{[OH_i\,]},$$

and hence $$\frac{[A_i^-]}{[A_0^-]} = \frac{1}{2}\left\{-\frac{[P]}{[A_0^-]+[OH_0^-]} + \sqrt{\left(\frac{[P]^2}{([A_0\,]+[OH_0^-])^2}+4\right)}\right\},$$

where the subscripts *i* and o indicate inside and outside concentrations respectively.

Before we consider the problem in detail we may point out that as long as P is great compared to $([A_0^-]+[OH_0^-])$ then $[A_i^-]/[A_0^-]$ approaches to $([A_0^-]+[OH_0^-])/[P]$, that is, a very small quantity.

Again we shall consider two cases where the ratio of $[A^-]/[HA]$ is varied. In the first [HA] is maintained constant and in the other α, the sum of [HA] and $[A^-]$, is constant. Having fixed $[H_0^+]$ the value of $[A_0\,]+[OH_0\,]$ is obtained and [HA], which is the same inside as out, is known. Then by trial a value of $[H_i^+]$ is chosen to give values of $[A_i\,]$ and [P] which satisfy the equation. If, however, $[OH^-]$ is negligible and $[A_i^-]$ negligible compared with [P], then $[H_0^+] = ([A_0^-].[H_i^+])/[P]$. When [HA] is constant

$$[A_0^-] = k_a[HA]/[H_0^+] \text{ and } [H_0^+] = (k_a[HA].[H_i^+])/[P]$$

and when α, the sum of [HA] and $[A^-]$, is constant

$$[A_0^-] = \alpha k_a/([H_0^+]+k_a) \text{ and } [H_0^+]([H_0^+]+k_a) = \alpha k_a[H_i^+]/[P].$$

Having fixed values for $[H_i^+]$ values for $[H_0^+]$ are calculated and hence the ratio of $[A_i^-]/[A_0^-]$.

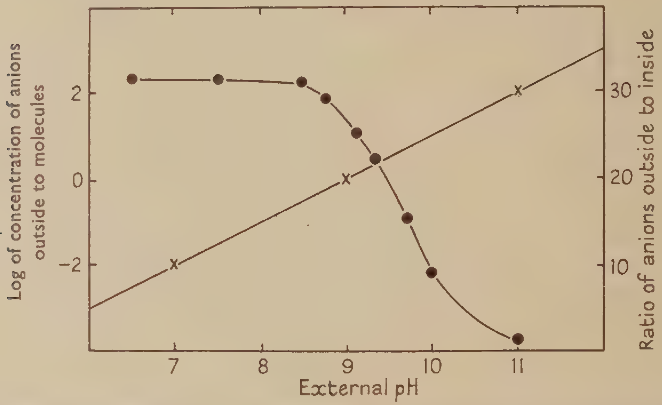

FIG. 1. Effect of external pH on the distribution of mobile anions of a weak acid when the concentration of molecules is constant. × logarithm of ratio of concentration of anions outside to molecules; ● ratio of concentrations of anions outside to inside.

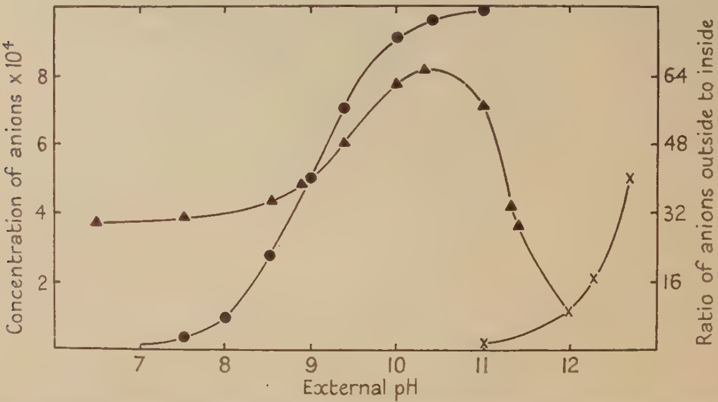

FIG. 2. Effect of external pH on the distribution of mobile anions of a weak acid when the external concentration of molecules plus anions is constant. ● concentration of anions outside; × concentration of anions inside; ▲ ratio of outside to inside.

In Fig. 1 the results are given for a system where $[HP]+[P] = \pi = 10^{-1}$ and k_p the acidic dissociation constant is 10^{-8}, and $[HA] = 10^{-3}$ with its dissociation constant $k_a = 10^{-9}$ while for Fig. 2 $\alpha = [HA]+[A_0^-] = 10^{-3}$.

Where [HA] is maintained constant outside while $[A_0^-]$ is increased the concentration of [HA] in the tissue will also remain constant, while the ratio of $[A_i^-]/[A_0^-]$ will increase towards unity. In the example chosen it is only

1/27 when $[A_0^-]$ has been made as big as [HA]. This fraction will be smaller the smaller the value of [HA] and the smaller k_a. For this example we should have to make $[A_0^-]$ nearly ten times as great as [HA] before $[A_i^-]$ reached equality with [HA]. Hence a negligible change of the effect of the weak acid when the external concentrations of the ion A^- is considerably increased (the concentration of molecules HA remaining constant) need not be evidence that the ions are ineffective in depressing the activity of X.

When [HA] falls $[A_0^-]$ is increased so that the total is constant; the value of [HA] inside will be equal to that outside, while the value of $[A_i^-]$ will rise with $[A_0^-]$ but relatively less rapidly at first, so that $[A_i^-]/[A_0^-]$ falls. In the example chosen when [HA] has fallen to 29 per cent. of the total, and $[A_0]$ is 2·4 times as big as [HA], $[A_i\ ]$ has risen to only 5 per cent. of the value of [HA] and is little more than 2 per cent. of the value of $[A_0^-]$. Even at $pH_0 = 11$, when 99 per cent. of the acid outside is in the ionized state the concentration of ions inside is little over 2 per cent. of that outside. Over this range no conclusions could be drawn about the relative effectiveness of the ions A^- and the molecule HA. To raise the value of $[A_0\ ]$ to $0{\cdot}5\times10^{-3}$, half of the total of $[HA]+[A_0^-]$, the external pH has to be increased to 9, but to bring $[A_i^-]$ to this value the external pH must be increased to as much as 12·69.

Over the range of pH_0 7 to 11 the change in the depressant effect will differ little for our three possibilities of p. 264; the amount of the difference will depend upon the relative value of [HA], at its maximum of 10^{-3}, to k_A. If the activity of X is proportional to that free from depressant and $k_A \gg 10^{-3}$, then when pH_0 is 11 the depressant effect will have fallen to the same extent that [HA] has, that is to 1 per cent. of that when pH_0 is 7 for both (*a*) and (*b*) and to 3 per cent. for (*c*). If k_A is only 10^{-4}, then for (*a*) the depressant effect will have fallen to 1/10 of that at pH 7, for (*b*) to 1/12, and for (*c*) to 1/4.

Hence with k_A relatively large it is difficult to distinguish between the three possibilities and when k_A is smaller then the ratio of depressant effect to concentration rises for all three possibilities; in other words, the rise of the ratio does not necessarily indicate that the ions are effective as depressants.

If only divalent cations are present then

$$\frac{[H_i^+]^2}{[H_0^+]^2}=\frac{[A_0^-]^2}{[A_i^-]^2}=\frac{[OH_0^-]^2}{[OH_i^-]^2}=\frac{[P]+[A_i^-]+[OH_i^-]-[H_i^+]}{[A_0^-]+[OH_0^-]-[H_0^+]}.$$

It can readily be shown that for a given value of $[H_0^+]$ the ratio of $[A_i^-]/[A_0^-]$ will be greater than when only univalent cations are present. For example the ratio of less than 1/30 from pH_0 6·5 to 8·5 for univalent becomes 1/10 for divalent cations, and whereas for univalent cations the ratio approaches $([A_0^-]+[H_0^+])/[P]$ when [P] is great compared with $[A_0^-]+[H_0^+]$, for divalent cations the ratio approaches $\sqrt{\{([A_0^-]+[OH_0^-])/[P]\}}$. Fig. 2 shows a ratio of $[A_i\ ]/[A_0^-]$ of 1/47 when the external pH = 11 and 1/9 at pH = 10; for divalent cations the ratios are 1/7 and 1/3. If both univalent and divalent cations are present the values for $[A_i^-]/[A_0^-]$ would be intermediate.

The substitution of bivalent for some or all of the univalent cations does not alter the nature of our conclusions, but our consideration does show that, if the tissue is placed in a relatively large bulk of outside solution, the substitution of a divalent cation for a univalent in the external solution could have a marked effect by bringing $[H_i^-]$ nearer to $[H_0^-]$ and so increasing the ratio of anions inside to out.

The above considerations suggest that the best procedure is to maintain [HA] constant while $[A^-]$ is increased and argue for the ions A^- competing with HA for X, with only XHA metabolically ineffective if depressant effect decreases as $[A^-]$ is increased, for both XA and XHA being ineffective if the depressant effect increases, but suspend judgement until there is evidence about the concentration of A^- inside if there is no change of depressant effect.

Studies in Stomatal Behaviour

VI. AN INVESTIGATION OF THE LIGHT RESPONSES OF WHEAT STOMATA WITH THE ATTEMPTED ELIMINATION OF CONTROL BY THE MESOPHYLL

PART 2. *Interactions with External Carbon Dioxide, and General Discussion*

O. V. S. HEATH AND J. RUSSELL

Research Institute of Plant Physiology, Imperial College of Science and Technology, London, S.W. 7

Received 15 October 1953

SUMMARY

As in the previous investigation (Part I), stomatal responses in wheat were followed by means of porometer readings; between these, air was forced through the stomata to sweep out the substomatal cavities. Instead of forcing the whole of a relatively slow air-flow in through the stomata, however, a fast flow was swept across the leaf surface and a portion of this passed into the intercellular space system. This helped to maintain the desired carbon dioxide content close to the stomatal openings and the stomata could thus be treated with different carbon dioxide concentrations. A factorial experiment was carried out in which 6 carbon dioxide concentrations from zero to 0·084 per cent. were combined with 3 light intensities from 90 to 800 f.c., the 18 treatments being replicated 6 times in three '6 × 6 latin squares'. A supplementary experiment in which the same 6 carbon dioxide treatments were given in darkness was carried out in duplicate. The results confirmed previous findings in that the stomata showed no appreciable response to variation of external carbon dioxide supply between zero and 0·01 per cent. concentration and that a large and highly significant light response was found at each of these two carbon dioxide levels. This is held to indicate the existence of a light effect (direct or indirect) not operating by reduction of the internal carbon dioxide content of the guard cells. On the assumption that the rates of air flow through the stomata were adequate, the combined results of the two experiments are interpreted mainly in terms of two direct effects of light upon the stomata, one depending on photosynthetic reduction of carbon dioxide in the guard cells and the other, mentioned above, not so operating. To these must be added the indirect effect responsible for the 'transmission' described in Part 1, which might possibly account for the whole of the second direct effect postulated. Finally, if the rates of flow were in fact grossly inadequate, the trends attributed to the first direct effect mentioned above might have been due to indirect control by the mesophyll via the intercellular space atmosphere. Such control must account for part of the light responses which occur under natural conditions. The natural light response is thus made up of at least two and more probably four components—two direct and two indirect, in each case one operating by the internal carbon dioxide and the other not. A highly significant diurnal rhythm of stomatal movement under constant illumination and temperature is shown to occur in wheat. The measure of stomatal resistance used (log resistance per 10,000 stomata) is discussed, and it is concluded that although it is empirical a much more satisfactory measure is not at present available.

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INTRODUCTION

THE previous paper of this series (Heath & Russell, 1954) deals with an experiment in which a light response was found in wheat stomata although the substomatal cavities were continuously swept between porometer readings with carbon dioxide free air. This, in conjunction with the earlier findings of Heath and Milthorpe (1950), is held to be evidence that the response was not dependent on reduction of the internal carbon dioxide content of the guard cells. Whether the light acted only indirectly on the guard cells by a stimulus transmitted from other cells (but not via the intercellular space atmosphere) or whether there was also a direct effect of light on the guard cells themselves was left undecided by the experiment in question. Evidence of such transmission of a light effect from one part of the leaf to another, even for a length exceeding 1·7 cm., was, however, obtained. In the present paper a larger and more comprehensive factorial experiment along similar lines is described. In this, different known carbon dioxide concentrations were used in the attempt to analyse the interaction of light intensity with carbon dioxide supply.

The literature was reviewed in the previous paper.

METHODS

The same plant material was used as in the previous experiment and the apparatus differed only in a few details; the previous paper should be consulted.

In view of the observed transmission of a light effect from one part of the leaf to another the use of one pair of leaf chambers as a 'control' was discontinued and a single pair of chambers was used on each leaf. By the use of two pairs of chambers, now quite independent as shown in Fig. 1, it was possible to carry out two treatments simultaneously on different plants, thus approximately halving the time necessary for a large experiment.

In the previous work the side walls ('washers') of the chambers were formed of a gelatine-glycerine mixture (Heath and Milthorpe, 1950), but, owing to the high humidity and high pressures used, breakdown of the walls sometimes occurred, especially with long exposure to 1,000 f.c. For the present experiments side walls of latex rubber were made as follows: A brass replica of the washer was first placed in position above the grooves in the perspex block and some of the gelatine-glycerine mixture in a molten state was poured round it and allowed to set. Removal of the brass model then left a mould, into which ammonia-preserved latex solution was poured and left to solidify overnight. The gelatine could then be stripped away, leaving the washer in position. A flat upper surface to each washer was next obtained by adding a thin layer of latex solution to each and placing the two chambers together with a glass slide between; this also ensured that the surfaces were exactly parallel. Finally the edges were trimmed where necessary with fine-pointed scissors. Dusting with talc was used to prevent the two washers from sticking together

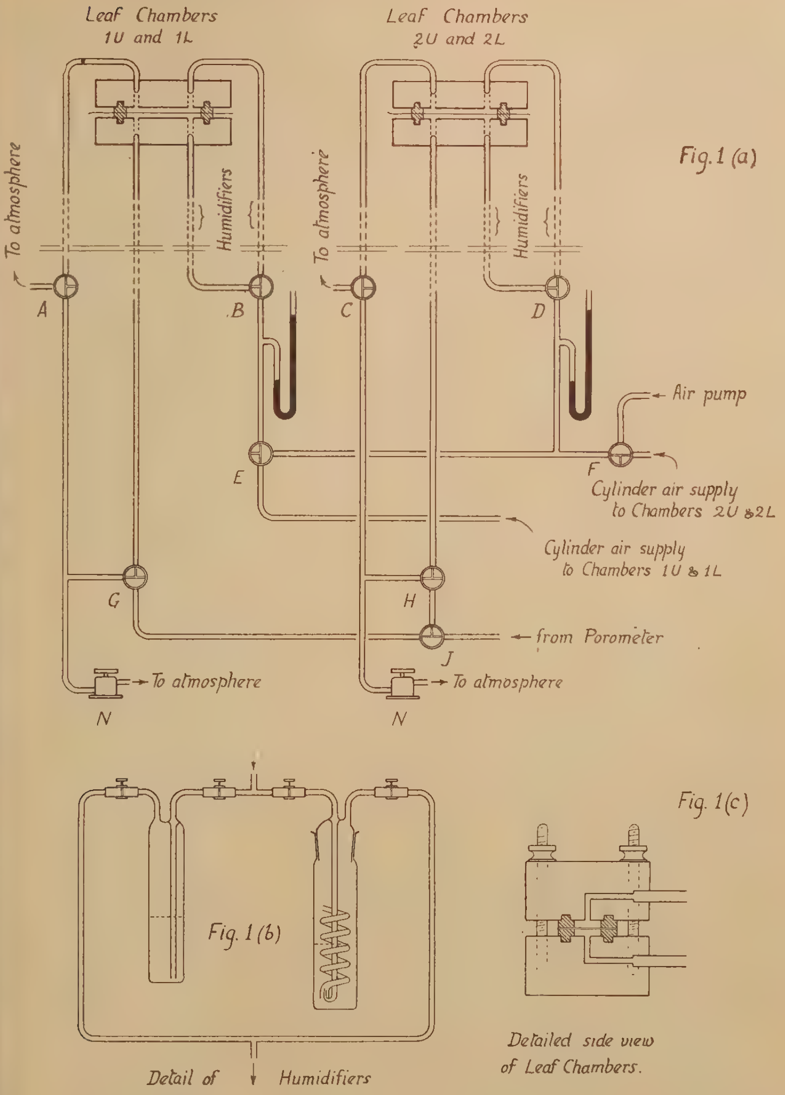

FIG. 1. *a.* Diagram showing method of control of air supplies to the leaf chambers. The portion of the diagram above the broken lines refers to the interior of the plant chamber. The taps are set for 'over-through' air-flow. For further explanation see text. *b.* Detail of humidifiers. The spiral bubbler contains saturated salt solution and the other bubbler distilled water. *c.* Transverse section through a pair of leaf chambers.

in use. In addition to being very durable and capable of repeated use, these latex washers gave an air-tight joint on the wheat leaf without the use of any grease or 'luting-wax'—a considerable advantage, especially under conditions of 'through' air-flow when the usual vaseline-beeswax mixture tended to spread, particularly on the upper leaf surface. Latex washers could be made narrower than those of gelatine without risk of breakdown and hence a higher rate of air-flow through the leaf be obtained for the same pressure.

In some treatments of the present experiments it was desired to maintain in the substomatal cavities carbon dioxide concentrations considerably in excess of 0·01 per cent., and the method of control used previously would have demanded an impracticably high rate of flow through the leaf, requiring excessive pressures. This consideration arose from the much higher rate of assimilation than respiration and the very rapid diffusion of CO_2 over the short paths from the stomata to the mesophyll cells. As a compromise, therefore, it was decided to sweep a rapid flow of air *over* the leaf surface, in an attempt to maintain the desired concentration as close as possible to the stomatal apertures, and to force a portion of this in *through* the stomata with the maximum pressure that appeared practicable. A flow of 8 l./hour of air at the known CO_2 concentrations was therefore fed into each pair of chambers, and with the taps set as shown in Fig. 1, a pressure of 5 cm. of mercury was maintained in the chambers by adjustment of the needle-valves *NN*. The proportion of the total flow constituting the 'through' flow thus varied with stomatal aperture. Its volume rate could be estimated by closing the needle-valve *N* completely and finding the flow-meter readings corresponding to a pressure of 5 cm. of mercury. This 'through' rate appeared to depend more on the width of washer than on stomatal resistance; the lowest value measured (in preliminary determinations) was 210 ml./hour. The adequacy of this procedure is considered below (pp. 282–7) in connexion with the interpretation of the results.

In the early part of the present experiment (first four 2-day periods, Table II, p. 274) a relative humidity of 50 per cent. in the air-flow was successfully used as previously. At the beginning of May 1951, however, possibly owing to a seasonal change in growth conditions, only limited stomatal opening could be obtained with 50 per cent. R.H. An increase of humidity to 75 per cent. (saturated sodium chloride) restored the magnitude of opening and time of response formerly obtained with 50 per cent. R.H., and this was used in all subsequent experimentation.

It was thought possible that the light effect found in the previous experiment might have been in part attributable to changes of leaf temperature, and the temperatures at the leaf surfaces under the various experimental conditions were therefore estimated with a thermocouple. This was made of 0·002 in. diameter silk-covered copper wire and copper-nickel wire, with a small piece of silver foil soldered to the tip to give good thermal contact with the lower leaf surface in the chamber. Calibration of the thermocouple gave a good linear response over a range of 8° C., with 0·97 cm. on the galvanomete scale per ° C. The leaf temperatures for the upper and lower surfaces under the various experimental conditions are shown in Table I. It was intended only to measure the temperatures for the lower leaf surface, but after the first series of determinations it was found that the thermocouple had pierced the leaf and rested flush with the upper surface. Although the light- and heat-absorption of the leaf and the thermocouple must have differed, and the flow distribution of air through the leaf would be changed by the piercing of both surfaces, these data for the upper surface are presented as approximations; the values are probably too low. During all the determinations readings were continued under each set of experimental conditions until successive readings showed no systematic change. In one instance seven readings were taken during a period of 75 minutes and showed a range of only 0·1° C., indicating that the temperature fluctuations of about 0·5° C. in the plant chamber were considerably damped down in the leaf chamber and of negligible importance. The tabulated data show that the change in lower leaf surface temperature due to the different light intensities was of the order of 2° C. with 0·25 l./hour 'through' air-flow as in the previous experiment and 1·5° C. with 8 l./hour 'over-through' air-flow as in the present investigation. For the upper leaf surface the ranges were apparently slightly greater. These temperature differences were thought to be of negligible importance.

TABLE I

Temperature (° C.) at leaf surface in leaf chambers

Air flow	Surface	Light intensity (f.c.)		
		90	270	800
0·25 l./hour 'through'	Upper	28·30	29·70	30·85
	Lower	27·05	27·65	28·80
8 l./hour 'over-through'	Upper	26·15	27·15	27·80
	Lower	26·05	27·05	27·50

In the present experiments, unlike the previous one, the light comparisons were between different leaves and it was therefore important to correct the porometer results for differences in area and stomatal numbers (cf. Heath and Russell, 1954, p. 10). The area of leaf enclosed in each pair of chambers was cut out with a razor-blade, following the faint impression left by the talc from the washers, and was preserved in 40 per cent. alcohol. Subsequently counts were made of the number of stomata on each surface. The average value of the ratio (α) of upper to lower stomata was found to be 1·31. From the porometer data values of $\log_{10}$ (resistance per 10,000 stomata) were calculated as

$$\log_{10} R_{10,000} = \log_{10} R_{\text{observed}} + \log_{10}\left(\frac{n_u n_l}{n_u + n_l} \times \frac{10{,}000}{5671 \times 4329}\right),$$

where n_u and n_l are the observed stomatal numbers on the upper and lower surfaces respectively, and 5,671 and 4,329 are the corresponding numbers to give a total of 10,000 and a ratio of 1·31. The assumptions implicit in the use of this expression are given in Heath and Milthorpe (1950) and discussed below (p. 289).

THE 'LIGHT × CO_2' EXPERIMENT AND 'DARK' EXPERIMENT

(11 January to 8 June and 27 to 28 June 1951)

1. *Design of experiments.* The 'light × CO_2' experiment was intended to investigate the relations of light and carbon dioxide in controlling stomatal movement with known carbon dioxide concentrations maintained as close as possible to the guard cells by means of a combination of 'over' and 'through' air-flow (p. 272). For this purpose six carbon dioxide concentrations (0·000, 0·010, 0·017, 0·029, 0·049, and 0·084 per cent.) were combined with each of the same three light intensities as in the previous experiment to give eighteen treatments in all. A separate 6×6 latin square was constructed (Fisher and Yates, 1948) for each light intensity, the rows of the square corresponding to different leaves and the columns to order of treatment with the various carbon dioxide concentrations. The six carbon dioxide treatments constituting one row were performed on one leaf at one light intensity: three treatments on each of two successive days.

As mentioned earlier, the two pairs of chambers were used independently on corresponding leaves of different plants, and this enabled a 'row' at each of two of the three light intensities to be completed in each 2-day experimental period. Nine such periods were thus needed to complete the three 6×6 squares. The light intensities were distributed among the 2-day periods as shown in Table II.

TABLE II

'Light × CO_2' Experiment 1951. Experimental periods and light treatments in f.c.

Period No.	1	2	3	4	5	6	7	8	9
Dates . .	11–12/1	22–23/4	25–26/4	28–29/4	1–2/5	7–8/5	10–11/5	3–4/6	7–8/6
Light treatments . .	800 90	90 800	270 90	800 270	800 270	800 270	800 90	90 270	270 90

With the exception of Period No. 1, the plants were given 9½ hours' darkness before each day's experimentation, i.e. a 14½-hour daylength. In the exceptional case continuous light was given and the plants had thus received some 60 hours' illumination by the end of the second day; the results of the second day appeared comparable with those of the first and also with the other periods, however.

To provide further information on some questions arising from the 'light × CO_2' experiment, an additional 'dark' experiment was performed, giving the same six carbon dioxide concentrations in darkness. The six treatments were carried out in reverse order upon two different leaves simultaneously (Table III).

TABLE III

'Dark' Experiment 1951. Order of carbon dioxide treatments (*vol. per cent.*)

	First day (27.6.51)			Second day (28.6.51)		
First leaf .	0·000	0·010	0·017	0·029	0·049	0·084
Second leaf .	0·084	0·049	0·029	0·017	0·010	0·000

This 'balanced' order was adopted in preference to a random distribution of the treatments in order to eliminate as much as possible of the diurnal effect (see below) from the treatment means and thus give the most precise estimation obtainable in so small an experiment of the 'dark' carbon dioxide responses of the stomata, on the assumption that the diurnal rhythm in darkness was similar in form to that found in light. This elimination of diurnal rhythm effects between treatments, however, results automatically in an increase of the variance within treatments and hence in an overestimate of the 'error' of the treatment comparisons. It follows that any treatment effects found significant are probably more significant than they appear to be.

The 'dark' experiment was of course analysed separately, but the results are discussed in relation to the 'light × CO_2' series, of which it formed an extension, although they are not *strictly* comparable, having been obtained at a later date and with only 2-fold instead of 6-fold replication.

2. *Results.* The analyses of variance for the two experiments are given in Tables IV and V, the mean values of $\log_{10} R_{10,000}$ found for each combination of carbon dioxide and light intensity (including the 'dark' experiment) are presented in Table VI and in a three-dimensional diagram in Fig. 2, and the mean curves for diurnal rhythm in the 'light × CO_2' experiment are shown in Fig. 3. The individual equilibrium values, both for these and the preceding 'light' experiment, are available in Russell (1951).

a. Diurnal rhythm. An extremely significant diurnal effect (order) was found in the 'light × CO_2' experiment, and this gave no indication of differing with light intensity, for the order × light interaction was negligibly small. Only the mean curves over all three light intensities are presented in Fig. 3, therefore. Each point is a mean of 18 values and is plotted at the average time of equilibrium. The dotted points represent the average values from the 18 initial porometer readings for the first or second day; as they are all at the same carbon dioxide concentration of approximately 0·03 per cent. they are not strictly comparable to the other values which are means of the equilibrium positions at six different carbon dioxide concentrations. However, they are included to give an indication of the morning values. In comparing these data, note that the vertical scale of Fig. 3 is double that of Fig. 2. Although there was no evidence of difference in *level* on the first and second day ('days' in the analysis), the *slope* (plus shape) of the curves showed a highly significant difference ('days × time' interaction). This took the form that the rhythm was much less marked on the second day, the stomata being less open at the first equilibrium time and showing a smaller closure which did not in fact reach significance (Fig. 3).

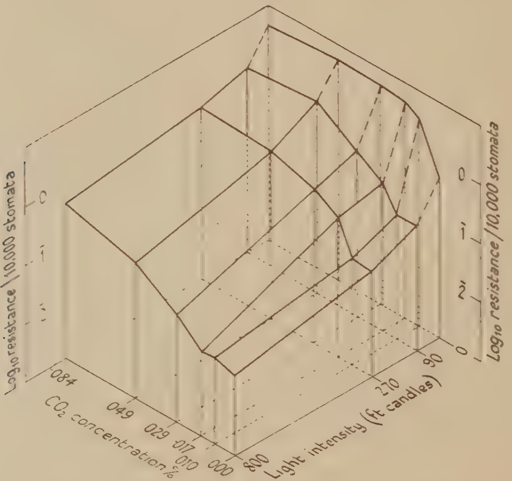

FIG. 2. Isometric projection of a 3-dimensional diagram showing the relations between light intensity and carbon dioxide concentration in affecting stomatal 'equilibrium values'. (Drawn to same scale as Fig. 2 of Heath and Russell, 1954.)

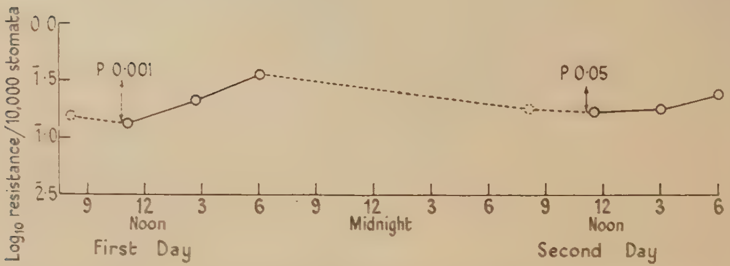

FIG. 3. Diurnal rhythm of stomatal movement in the 'light × CO_2' experiment.

b. Light. The average light effect in the 'light × CO_2' experiment only reached a level of significance between P 0·05 and 0·01, doubtless because the light comparisons were 'between leaves' and the appropriate error was thus based on the large and very significant leaf-to-leaf differences ('leaves', Table IV). If it be accepted that the 'over-through' air-flow maintained the carbon dioxide concentrations at the permeable parts of the guard cell surfaces substantially independent of changes in assimilation by the mesophyll cells, this

TABLE IV

'Light × CO_2' Experiment, 1951. Analysis of variance on values of $\log_{10} R_{10.000}$
(3 latin squares, each 6×6)

Variation	Degrees of freedom	Variance	*F*	*F* required for *P* 0·05	**P 0·01**	**P 0·001**
Total . . .	107	—	—	—	—	—
Squares (light) . .	2	6·4386	*4·70*	3·68	6·36	—
Rows* (leaves) . .	15	1·3701	**14·65**	—	—	< 3·3
Days . . .	1	0·103	1·10	4·00	—	—
Time . . .	2	0·675	***7·21***	—	4·98	7·76
Days × Time . .	2	0·705	***7·53***	—	4·98	7·76
Columns (order) .	5	0·5724	**6·12**	—	—	4·76
High v. low (CO_2') .	1	16·890	—	—	—	—
CO_2 increase (CO_2'') .	2	1·612	—	—	—	—
Interaction ($CO_2''\ '$) .	2	0·304	—	—	—	—
Carbon dioxide .	5	4·1443	**44·31**	—	—	4·76
Light × CO_2' .	2	0·016	—	—	—	—
Light × CO_2'' . .	4	0·033	—	—	—	—
Light × $CO_2''\ '$.	4	0·281	*3·00*	2·52	3·65	—
Light × carbon dioxide . . .	10	0·1286	1·37	> 1·92	—	—
Order × light . .	10	0·0002	—	—	—	—
Residue (Error) .	60	0·0935	—	—	—	—

* Error for squares (light).

TABLE V

'Dark' Experiment, 1951. Analysis of variance on values of $\log_{10} R_{10,000}$

Variation	Degrees of freedom	Variance	*F*	*F* required for *P* 0·05	***P* 0·01**
Total	11	—	—	—	—
Leaves . . .	1	0·0061	—	—	—
CO_2	5	0·3066	*9·66*	5·05	10·97
CO_2 × leaves (Error) .	5	0·0317	—	—	—

significant average effect provides confirmatory evidence for a light effect independent of intercellular-space carbon dioxide. The mean values of $\log_{10} R_{10,000}$ at the different light intensities were: 90 f.c., $\bar{1}$·68; 270 f.c., $\bar{1}$·42; 800 f.c., $\bar{2}$·85. The difference between the means at 90 and 800 f.c. was significant at the *P* 0·01 level, while that between 270 and 800 f.c. just failed to reach the *P* 0·05 level. The mean value for the 'dark' experiment was 0·53.

c. Carbon dioxide. The carbon dioxide effect in the 'dark' experiment nearly reached the *P* 0·01 significance level (Table V), and this was almost entirely due to the zero carbon dioxide value which was very significantly ($P <$ 0·01) lower than any other. The values are indicated in Table VI. In the 'light × CO_2' experiment the average carbon dioxide effect was of overwhelming significance (Table IV). The means are presented at the bottom of Table VI. It is seen that there is no good evidence of an effect of carbon dioxide between zero and 0·01 per cent., consistent with Heath and Milthorpe's finding, nor between 0·049 and 0·084 per cent. All other differences between carbon dioxide levels are highly significant.

TABLE VI

'Dark' and 'Light × CO_2' Experiments, 1951. Treatment means, $\log_{10} R_{10.000}$

	Percentage carbon dioxide					
Light (f.c.)	0·084	0·049	0·029	0·017	0·010	0·000
0	0·68	0·77	0·74	0·69	0·56	$\bar{1}$·74
(Significant differences P 0·05, 0·458; P 0·01, 0·718)						
90	0·16	0·26	$\bar{1}$·81	$\bar{1}$·54	$\bar{1}$·14	$\bar{1}$·17
270	$\bar{1}$·91	$\bar{1}$·86	$\bar{1}$·60	$\bar{1}$·44	$\bar{2}$·87	$\bar{2}$·81
800	$\bar{1}$·64	$\bar{1}$·33	$\bar{2}$·83	$\bar{2}$·45	$\bar{2}$·47	$\bar{2}$·39
(Significant differences P 0·05, 0·35; P 0·01, 0·47; P 0·001, 0·51)						
Mean (90–800)	$\bar{1}$·90	$\bar{1}$·82	$\bar{1}$·41	$\bar{1}$·14	$\bar{2}$·83	$\bar{2}$·79
(Significant differences P 0·05, 0·20; P 0·01, 0·27; P 0·001, 0·35)						

d. Carbon dioxide and light interaction. The total interaction of light and carbon dioxide (10 degrees of freedom) fails to reach significance in the 'light × CO_2' experiment (Table IV), possibly because the striking differences of the two end portions of the carbon dioxide curve at 800 f.c. from those at 270 and 90 f.c. are largely swamped by the generally parallel disposition of the curves. For theoretical reasons discussed below (p. 279) the differences in these end portions of the curves at the various light intensities are of primary interest, and the 10 degrees of freedom for interaction have therefore been subdivided so as to isolate this aspect. The method adopted treats the successive steps of carbon dioxide concentration as formally equal, but there does not appear to be any great objection to this. The carbon dioxide range is divided into 'high' (0·029 to 0·084 per cent.) and 'low' (zero to 0·017 per cent.), thus accounting for one degree of freedom (CO_2'). Two more degrees of freedom represent the average effect of CO_2 increase within these classes (CO_2'') and the interaction (CO_2''') accounts for the other two out of the five for carbon dioxide concentration. The interaction of light intensity with CO_2''' is found significant ($P <$ 0·05), showing that the difference in response to increase of carbon dioxide concentration in the 'high' and 'low' carbon dioxide ranges is not the same for all light intensities. It is therefore legitimate to apply the t test within these two ranges at the various individual light intensities.

There is little doubt that if the carbon dioxide curve obtained in the 'dark' experiment could be included in the same analysis with the main experiment, assuming it to be comparable, a significant over-all light and carbon dioxide interaction would be found.

Application of the t test (Table VI) shows that the response to change from 0·01 per cent. to zero carbon dioxide in the dark is significant at the P 0·01 level, as already mentioned. On the other hand, the very small responses over this range at the various light intensities of the ‘light × CO_2’ experiment do not even approach significance, nor does that between 0·01 and 0·017 per cent. at 800 f.c.; the stomatal closure over the latter range at 270 f.c. and 90 f.c. is in each case, however, quite significant ($P <$ 0·05 and $<$ 0·001 respectively). In the ‘high CO_2’ half of Table VI, at 800 f.c. the rise of $\log_{10} R_{10,000}$ between 0·049 and 0·084 per cent. does not quite reach the P 0·05 level, but a significance of $P <$ 0·001 is found for the response between 0·029 and 0·084 per cent.; at none of the other light intensities is there any appreciable response between 0·049 and 0·084 per cent., while even from 0·029 to 0·084 per cent. the significance only approximates to P 0·05 (270 f.c. and 90 f.c.) or is negligible (‘dark’ experiment). As far as light effects are concerned, attention should especially be called to the large and highly significant responses between 90 f.c. and 800 f.c. at both zero and 0·010 per cent. carbon dioxide ($P <$ 0·001).

DISCUSSION

1. *Evidence for direct effects of light upon the guard cells.* The main interest in the interaction of carbon dioxide and light as shown in Fig. 2 lies in the significant aspect isolated with the ‘light × CO_2′ ‴’ degrees of freedom, i.e. changes with light intensity of the differences in shape, and especially in flattening, of the carbon dioxide response curves at high and low carbon dioxide concentration. If horizontal, which cannot be proved, these flattenings will be independent of the measure of stomatal aperture; the shapes of the *light* response curves, though yielding the same information about the interaction, are less easy to discuss because of the empirical nature of the measure used. On the assumptions that the ‘dark’ and ‘light × CO_2’ experiments were truly comparable and that the ‘through-over’ air-flow was in all cases adequate in the sense set forth above (p. 276), the following interpretation of the results presented in Fig. 2 and Table VI is suggested.

The carbon dioxide operated mechanism becomes saturated with carbon dioxide at about 0·017 per cent. external concentration in darkness, at about 0·049 per cent. in 90 f.c. and at a somewhat similar level in 270 f.c.; in 800 f.c. light intensity the mechanism is apparently not saturated below 0·084 per cent. external carbon dioxide, for the curve is still rising up to that point. This trend is strongly indicative of photosynthesis in the guard cells, which increases with rising light intensity and reduces the internal carbon dioxide concentration corresponding to a given external concentration. In darkness the carbon dioxide produced in the guard cells by respiration is apparently so abundant, and resistance to outward diffusion so high, that with only 0·017 per cent. externally the mechanism is still saturated with carbon dioxide. In

800 f.c., on the other hand, even 0·084 per cent. external supply supplemented by the internal respiratory carbon dioxide is insufficient to maintain saturation, and with 0·017 per cent. externally the internal concentration is reduced to an effective 0·01 per cent. giving maximal opening.

So far as the authors are aware, this is the first experimental evidence that photosynthesis in the guard cells is concerned in the normal stomatal response to light (Heath and Russell, 1954, p. 3). The evidence in the present work, however, indicates that its effect depends upon the reduction of the internal carbon dioxide concentration and not to any important extent upon the production of carbohydrate, which was in effect the mechanism postulated by von Mohl (1856), except perhaps for the long-term maintenance of osmotic pressure. Otherwise, increasing the external carbon dioxide supply in light would tend to cause stomatal *opening* and this tendency would be most marked at the highest light intensity where photosynthesis was least light-limited. However, except from 0·010 to 0·017 per cent. carbon dioxide, the converse situation is seen: increase of carbon dioxide causes greater *closure* at 800 f.c. than at 270 f.c. or 90 f.c.

The data under discussion also give indications that the direct effect of light includes a second component which is *not* dependent on the photosynthetic reduction of the carbon dioxide in the guard cells: the approximately CO_2-saturated portions of the response curves between 0·049 and 0·084 per cent. external carbon dioxide show progressively much lower stomatal resistance as one passes from darkness to 90 f.c. and thence to 270 f.c. It seems improbable that so large a light response would be due to photosynthetic removal of internal carbon dioxide in a region where a reduction of the external carbon dioxide from 0·084 to 0·049 per cent. is virtually without effect. Furthermore, between 0·000 and 0·010 per cent., where external and therefore presumably internal carbon dioxide is apparently without effect, there is a highly significant light response from 90 to 800 f.c.; a similar and significant light response with CO_2-free air passing through the stomata was obtained in the 'light' experiment (Heath and Russell, 1954). In Heath and Milthorpe's (1950) preliminary work also, a light effect with either 0·00 or 0·01 per cent. carbon dioxide supplied to the leaf surface was indicated, though its significance could not be estimated.

In view of this second direct light effect, an alternative interpretation of the flattening of the curve from 0·017 to 0·010 per cent. at 800 f.c. is that even if the internal concentration was not reduced to the minimum at an external concentration of 0·017 per cent., the stomata were then as widely open as was mechanically possible. This suggestion is rendered plausible by the similarity to the maximum opening obtained by Heath and Milthorpe (1950), viz. about $\bar{2}\cdot26$ for $\log_{10} R_{10{,}000}$ as compared with $\bar{2}\cdot39$ in the present work.[1] This maximum opening obtained by Heath and Milthorpe, using 975 f.c. and a low rate of flow of dry air (2 l./hr.), remained constant at all carbon dioxide concentrations supplied from zero to 0·03 per cent. They attributed this apparent lack of carbon dioxide effect to excessive depletion of the external supply by photosynthesis, but it may have been in part caused by the stomata having reached the maximum possible aperture, owing to the direct light effect under discussion, even with 0·03 per cent. carbon dioxide in the air supply.

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[1] On the other hand, the maximum closure in darkness was much less complete than that at 275 f.c. in Heath and Milthorpe's experiments (see below, p. 288). This suggests that the flattening of the 'dark' experiment curve above 0·017 per cent. CO_2 was not due to the stomata being mechanically incapable of further closure.

The interpretation put forward in this section is thought to be the simplest hypothesis to account for the results presented in Fig. 2 and Table VI. Some considerations which must modify it in detail and may even affect its more general validity are discussed in the following section. In the interests of brevity the two direct light effects just postulated will be termed the 'first direct effect' and 'second direct effect', the former operating by photosynthetic reduction of the carbon dioxide content in the guard cells and the latter not so operating.

2. *Indirect light effects.* Indirect effects of light upon the guard cells, due to photosynthetic or other changes caused in the epidermal or mesophyll cells by light, may be transmitted to the stomata (*a*) via the intercellular space atmosphere or (*b*) along gradients from cell to cell.

a. The flow of air through the stomata in the present experiments was expressly intended to prevent the transmission of indirect light effects in this way and to substitute as far as possible known carbon dioxide concentrations at the guard cell surfaces for the unknown ones due to photosynthesis and respiration in the mesophyll cells, thus separating the effects of light and external carbon dioxide. In so far as the results can be taken at their face value the carbon dioxide responses found therefore illustrate, like those found by Heath and Milthorpe (1950) but rather more comprehensively, the indirect control of stomatal movement by changes of light intensity operating via the intercellular space carbon dioxide content. In the absence of a 'through' airflow, as under natural conditions and in Heath and Milthorpe's experiments, the response to increased light intensity must include in addition to the direct effects postulated in the preceding section some indirect effect due to reduction of intercellular space carbon dioxide content. Hence the response curves would follow a course not parallel to the light curves in Fig. 2 but more or less diagonally across the surface of the figure. This may partly account for the discrepancy in the shape of the light response curves between the present authors' experiments and those of Heath and Milthorpe: the former show a predominantly falling response to light with increasing light intensity (Fig. 2 and Heath and Russell, 1954, Fig. 2) such as might be expected on any hypothesis of the 'limiting factor' type, whereas the latter show a curvature in the opposite sense (Heath and Milthorpe, 1950, Figs. 7 and 8). In their experiments, however, the light treatments concerned were not only given on different leaves and at different times but were unreplicated; too much importance should not be attached to the shape of their light curves, therefore.

It would appear that if the intercellular space carbon dioxide has been reduced to 0·01 per cent. no further opening as an indirect carbon dioxide operated light response is to be expected, both because the stomata do not respond to reduction of external carbon dioxide below this level and because, if the results quoted by Heath and Russell (1954, p. 2) are of general application, a reduction below this level by photosynthesis cannot occur. For conditions of darkness, the curve in Fig. 2 might seem to suggest that *above* 0·01 per cent. concentration external carbon dioxide is almost without effect and therefore that there can be virtually no control by the intercellular space atmosphere. It should be remembered, however, that these are equilibrium values and the intercellular space carbon dioxide may have a larger effect on the *rate* of closure in darkness.

It is now necessary to consider how far the rates of 'through' air-flow were adequate to maintain near approximations to the desired carbon dioxide concentrations at the guard cell surfaces, for if very inadequate the trends attributed to photosynthesis in the guard cells may in fact have been indirect effects due to photosynthesis in the mesophyll.

For the 'light' experiment (Heath and Russell, 1954) in which a 'through' flow of 250 ml. hour of carbon dioxide free air was passed in through the stomata, it was thought to be reasonably certain that a virtually carbon dioxide free atmosphere was maintained in contact with the guard cell surfaces, and the effects of light independent of the intercellular space carbon dioxide content postulated in the paper cited may be accepted on these grounds. The highly significant light responses at both zero and 0·01 per cent. carbon dioxide supply in the 'light × CO_2' experiment may also be accepted as independent of external carbon dioxide concentration, for with such relatively wide stomatal openings the 'through' part of the air-flow must greatly have exceeded 250 ml. hour, while in the case of the 0·01 per cent. carbon dioxide supply the net uptake or output of carbon dioxide by the leaf presumably approximated to zero. In all these cases the data are thus consistent with the existence of a direct effect of light, and since they refer to the region 0–0·01 per cent., where changes of external carbon dioxide are apparently without effect, it must be presumed here to be of the second kind mentioned in the preceding section (p. 280).

In the 'dark' experiment also the carbon dioxide concentrations at the guard cell surfaces were presumably near approximations to those supplied in the air streams, for again the respiratory carbon dioxide would only account for a concentration of the order of 0·001 per cent. even with uniform mixing (cf. Heath and Russell, 1954, p. 4). It is with the higher carbon dioxide concentrations of the 'light × CO_2' experiment that doubt arises as to the adequacy of the 'through' rate of flow, for here the carbon dioxide was depleted by assimilation at a rate which must greatly have exceeded that of respiration, and owing to diffusion serious depletion may have occurred in the air supply even before it reached the inner surfaces of the guard cells. Unfortunately not much information is available for assimilation rates from 'through' air streams, the only published data being those of Heath (1951) for

Pelargonium and *Begonia*. These indicate that at high light intensity (2,450 f.c.) with a 'through' supply of up to about 15 mg./dm.^{2}/hour, all the carbon dioxide supplied was assimilated except for a residual 0·01 per cent.; with 30 mg./dm.^{2}/hour (initial concentration 0·184 per cent.) the residual carbon dioxide was of the order of 0·017 per cent. (Heath, 1951, Fig. 2). This, however, was for air which had passed laterally in the intercellular space system for *at least* 2 mm. (the width of the washers). In the present experiments it is the depletion of carbon dioxide at the stoma rather than at the mesophyll that is of importance, but since the path from the stoma to mesophyll cell is short the difference in concentration at the *inner* stoma mouth and at the surface of the assimilating cell will not be great.

Some estimates of the possible extent of the carbon dioxide depletion at the inner mouths of the stomata have been made as follows: First, assuming that the stomatal pores had a long axis ($2a$) of 32 μ and a depth (l) of 3 μ, obtained from measurements on microtome sections, a value for the short axis ($2b$) was found which gave l as 3 μ in the equation

$$R_1 = \frac{l_{\mathrm{eff}}}{r_{\mathrm{eff}}} = \frac{(l+2x)0{\cdot}9}{(2\pi ab/p)^4}.$$

Here R_1 is the resistance per stoma obtained from porometer measurements, l_{eff} and r_{eff} are the 'effective length' and 'effective radius', p is the perimeter and x the 'end correction'[1] for an elliptical tube of semi-axes a and b. The estimates of pore width so obtained were not unreasonable and ranged from 11·6 μ for the lowest individual measured resistance (with 800 f.c. light intensity and carbon dioxide free air) to 1·2 μ for the highest (90 f.c. and 0·049 per cent. carbon dioxide). For the lowest equilibrium resistance found using air of more than 0·01 per cent. carbon dioxide (800 f.c. and 0·017 per cent.) the estimated short axis was 9·6 μ (Table VII, line 4). The greatest pore width actually seen as yet by direct microscopic observation of the living wheat leaf is 10 μ, but this is at the narrowest part of the stomatal tube and the effective width, even for viscous flow, must be appreciably greater than this. Next it was assumed that the stoma had these dimensions and that the assimilating mesophyll cells were 30 μ distant, forming the surface of a hollow half-cylinder 32 μ long and $(30+b)$ μ in radius (Fig. 4). The partial pressure of carbon dioxide supplied, p_1 atmospheres (Table VII, line 1), was assumed to be maintained in the chamber, while that at the mesophyll (p_2) was taken to be $1 \times 10^{\ 4}$ atm. as the worst case. The total resistance to diffusion was taken as

$$Z = \underbrace{\left\{\frac{(l+x)0{\cdot}9}{A}\,\frac{RT}{DM}\right\}}_{\text{stoma}} + \underbrace{\left\{\frac{L}{A_{lm}}\,\frac{RT}{DM}\right\}}_{\text{sub-stomatal cavity}^{2}} \text{sec. atm./g,}$$

---

[1] Calculated by H. L. Penman (personal communication). This end correction is somewhat smaller than that for a cylindrical tube of the same cross-sectional area, especially at small values of a.

[2] The authors are indebted to Dr. D. C. Spanner for suggesting this treatment of the substomatal cavity resistance.

where x = the 'end correction' and the 'effective length' $l-x$ is reduced by 10 per cent. following Penman and Schofield (1951, p. 121); $A = \pi ab$, the area of the pore; L is the distance from the stoma to the mesophyll (30 μ in Fig. 4); A_m is the 'logarithmic' mean of the surface areas of two coaxial half-cylinders of length $2a$, one of area A_1 and radius b and the other of Area A_2 and radius $L-b$, i.e. $\frac{A_2-A_1}{\log_e A_2/A_1}$; R is the gas constant, T the absolute temperature, while D and M are the diffusion coefficient and molecular weight for carbon dioxide.

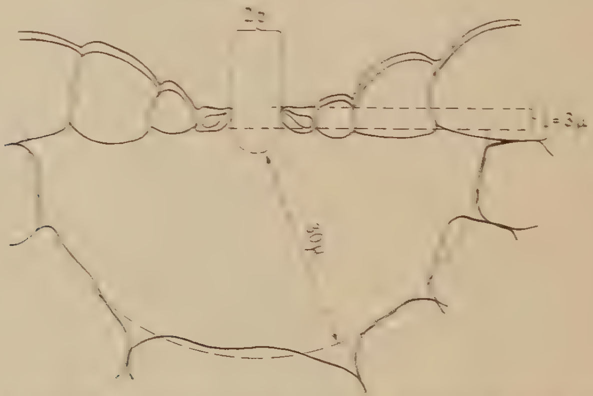

Fig. 4. Diagram of dimensions used in estimating depletion of carbon dioxide supply by assimilation in the mesophyll.

The rate of gas supply by diffusion alone (i.e. from an 'over' air-flow only) was given by

$$Q = \frac{p_1-p_2}{Z}\ \text{g./sec.}$$

Hence the ratio of the rate of 'available' carbon dioxide supply by a 'through' flow of 210 ml./hour (as the worst case) to Q was obtained (Table VII, line 7).

From the estimated resistances to diffusion for the stoma (Z_s) and sub-stomatal cavity (Z_c) the partial pressure of carbon dioxide (p_i) just inside the stoma, due to diffusion alone, was obtained from

$$p_i - p_2 = \frac{(p_1-p_2)Z_c}{Z_s-Z_c} \quad \text{(Table VII, line 5).}$$

Hence also the ratio of the 'available' carbon dioxide just inside the stoma $(p_i - 1{\cdot}0 \times 10^{-4} = p_i - p_2)$ to that in the chamber $(p_1 - 1{\cdot}0 \times 10^{-4} = p_1 - p_2)$, with 'over' air-flow only, was found (Table VII, line 6).

TABLE VII

'Light × CO_2' Experiment. Estimates of depletion of carbon dioxide supply at inner stoma mouth due to diffusion, assuming $p_2 = 1\cdot0\times10^{-4}$ *atm.* CO_2 *at the mesophyll*

Light (f.c.)	270				800			
1. p_1 (atm. × 10^{-4}) . .	8·4		1·7		8·4		1·7	
2. mg./dm.^{2}/hr. at 8 l/hr. .	1,115		228		1,115		228	
3. Max. and min. R_1 (cm.$^{-3}$ × 10^9) . . .	2,480	164	1,270	69·8	2,920	18·4	130	6·22
4. Estimated $2b$ (μ) . .	1·6	3·6	2·0	4·6	1·6	6·9	3·8	9·6
5. Estimated p_i (atm. × 10^{-4}) .	2·89	3·30	1·19	1·23	2·88	3·59	1·22	1·26
6. $(p_i-p_2)/(p_1-p_2)$ . .	0·26	0·31	0·27	0·33	0·25	0·35	0·32	0·37
7. Ratio of 'available' CO_2 supplied by 'through' flow of 210 ml./hr. to Q . .	0·088	0·056	0·078	0·049	0·090	0·040	0·054	0·033

The values presented in lines 6 and 7 of Table VII suggest that depletion of the carbon dioxide supply at the inner stomatal mouth was on the whole extremely serious. Even if p_2 is taken as $2\cdot0\times10^{-4}$ atm., and since 210 c.c./hour of air containing $8\cdot4\times10^{4}$ atm. is equivalent to 31/mg./dm.^{2}/hour of carbon dioxide, the result quoted on p. 283 makes this seem reasonable for the highest concentration, the improvement is not very great. If such depletion really occurred it might well produce trends of stomatal resistance such as those which have been attributed to photosynthesis in the guard cells: in darkness the concentration at the inner stoma mouth (p_i) would be sensibly the same as that supplied (p. 282 above), but with increasing light intensity increased assimilation would cause a fall in p_2[1] and hence in p_i and therefore also in the *internal* concentration corresponding to a given concentration supplied.

The values in Table VII present the worst case, however, and the 'second direct effect' of light (p. 281) would operate against this trend, for stomatal opening due to this direct effect would increase the 'through' rate of flow and so reduce the depletion.

The following argument may be advanced against the above suggestion that the apparent evidence for photosynthetic reduction in the guard cells of the internal carbon dioxide is an artefact due to depletion in the supply. For assimilation in the *mesophyll* cells, carbon dioxide must have been severely limiting over most of the ranges of light and carbon dioxide supply studied. Thus in Maskell's (1928) experiments with cherry laurel the 'low' light was about 0·14 × sunlight (say 300 f.c.) and to get 'light-limited' assimilation 2 to 3 per cent. carbon dioxide was needed according to stomatal aperture. Therefore changing light intensity, at least between 270 and 800 f.c., should have had little effect on intercellular space carbon dioxide content and hence little effect on the value of p_1 at which the carbon dioxide operated mechanism

[1] No allowance has been made in Table VII for a difference in p_2 at 270 and 840 f.c.

became saturated. The movement of the 'carbon dioxide saturated' portion of the curve towards the higher end of the carbon dioxide scale in Fig. 2 is, however, much greater between 270 and 800 f.c. than between 90 and 270 f.c. This suggests that the photosynthesis concerned is in the guard cells rather than in the mesophyll and that in the former the compensation point is higher and the 'light-limited' region extends to much higher light intensities. Note that these will be affected by the supply of respiratory carbon dioxide from the adjacent epidermal cells, which are free of chloroplasts in wheat: this will tend to make the stomatal response to increasing light intensity more gradual than it otherwise would be, and in darkness it will help to keep the stomata relatively closed even with very low external concentration (as in Fig 2).

An experimental check on this argument would have been most desirable. This would have involved collecting the 'through' air-flow as it emerged from the leaf and estimating its carbon dioxide content. If there was not much light effect upon the assimilation rate so measured between, say, 270 and 800 f.c., i.e. if assimilation was severely 'CO_2-limited', the changes observed must be due to assimilation in the guard cells. This would have added greatly to the difficulties of a technique already by no means easy, however, and a simpler experimental check would have been to try different pressures (and hence change the rates of 'through' flow) near to the value actually used. If these changes made no difference to stomatal resistance the through flow could be assumed adequate. Owing to the long delay in obtaining 'equilibrium' values such determinations could not have been carried out during the actual experiment, and a series of subsidiary determinations after its completion was prevented by the departure of the junior author to take up other commitments. A probable complication which might well arise in such an experimental check is that due to the drying effect of the air stream. Wheat stomata show closure in response to drying (p. 272, and Heath and Milthorpe, 1950) and hence increased 'through' flow with any humidity below saturation would be expected to cause closure.

Drying effects of the kind just discussed must have distorted the curves in the 'light × CO_2' experiment, and falling rates of 'through' flow with stomatal closure must have resulted in less drying as well as more depletion of carbon dioxide; the closure must therefore have been less marked than would have been obtained with constant flow. The use of constant 'through' flow was only abandoned in this experiment because of the technical difficulties involved, but as is seen these are replaced by difficulties of interpretation. An alternative method of carrying out the experiment which might have provided less equivocal results would have been to draw air through the leaf *into* the leaf chambers and measure the carbon dioxide content of the emerging air, as in Heath (1939). This would have given the concentration at the inner mouths of the stomata (with a sufficient flow rate), but the difficulties of maintaining constant values in definite steps and at different light intensities appear almost insuperable.

It seems likely that the 'over' air-flow in the 'light × CO_2' experiment was fairly adequate: 7·8 l./hour of air containing 0·029 per cent. would represent 379 mg. CO_2/dm.^{2}/hour. If the assimilation from an 'over' air-flow of this rate and concentration were as high as, say, 40 mg./dm.^{2}/hour, even at the exit end of the chamber the concentration would only be reduced to 0·026 per cent.; this is an average for the whole cross section of the leaf chamber, and at the stoma mouth it would be lower. It would seem, then, that even though the depletion at the inner stoma mouth with a 'through' flow of, say, 210 ml./hour were very severe, that at the outer surface or even perhaps half-way up the stomatal tube might not be excessive. This raises the question of where carbon dioxide enters the wheat guard cells. It has been assumed that, as has been shown experimentally for *Pelargonium*, the carbon dioxide can only operate from the sub-stomatal cavity, but in view of the thin walled ends of the guard cells in wheat it may be able to enter them from the outer surface also. This suggestion is rendered the more plausible by the fact that wheat stomata show a marked closing response to a dry external atmosphere (Heath, 1950; Heath and Milthorpe, 1950) whereas *Pelargonium* does not. If, even with wide open wheat stomata, the 'available' concentration at the inner stomatal mouth is only one-third of that outside (Table VII, line 6), with an 'over' flow of 0·03 per cent. (0·02 per cent. 'available') there will only be 0·017 per cent. (0·007 per cent. 'available') at the inner mouth. It seems remarkable that this should have the great closing effect as compared with 0·01 per cent. found by Heath and Milthorpe, again suggesting that wheat stomata respond to carbon dioxide exterior to the leaf. If they do, the results in Fig. 2 may be taken more or less at their face value and the interpretation in the previous section accepted.

b. Effects transmitted from cell to cell. Light effects due to carbon assimilation and transmitted by gradients of carbon dioxide concentration may first be considered. Since the subsidiary and other epidermal cells are apparently free of chloroplasts they are presumably not responsible for the trends attributed to photosynthesis in the guard cells in Section 1 above; it might be argued that carbon assimilation in the even more remote mesophyll cells was concerned, but as pointed out in the previous paper the diffusion paths from the guard cells to these are long, and in the absence of evidence the simpler hypothesis should be preferred.

Although the trends attributed to the first direct effect (p. 281) are thus for the present not thought to be due to an indirect effect transmitted from cell to cell by carbon dioxide gradients, the evidence for a 'transmission effect' presented in the previous paper suggests that an indirect effect transmitted by some other agent accounts for at least part of those attributed to the 'second direct effect' postulated in Section 1.

There is also another way in which this 'transmission effect' may have modified the results as seen in Fig. 2. In the 'light × CO_2' experiment only the area of leaf in the chambers was maintained at the three experimental light intensities, the rest of the leaf being at about 800 f.c. throughout; in the 'dark' experiment the whole leaf was in darkness. Owing to the transmission of light effects it may thus be expected that the light response to 270 f.c., and even more that to 90 f.c., was somewhat overestimated. Therefore, if the whole leaf had been evenly illuminated in all treatments, the evidence for the 'second direct effect' would presumably have been reduced by a smaller light effect between zero and 270 f.c. in the 'carbon dioxide saturated' region of the curves, but on the other hand it would have been correspondingly increased by a larger light effect from 90 to 800 f.c. in the region from 0·010 to 0·000 per cent. carbon dioxide. This is assuming that the transmission effect would not modify the carbon dioxide responses, and since there is as yet no evidence that such modification occurs, the discussion based on the shapes of the carbon dioxide response curves may stand for the present. It may be noted that in Heath and Milthorpe's (1950) experiments the whole leaf was evenly illuminated and over the more limited ranges explored very similar carbon dioxide and light effects were obtained, including a large light effect in the carbon dioxide range 0·00 to 0·01 per cent.

3. *Diurnal rhythm of stomatal movement.*—The diurnal effect (order) in the 'light' experiment did not even approach significance, possibly because of the wide scatter in the times of equilibrium coupled with the fact that it was only based on results from six leaves. In the 'light × CO_2' experiment, although the times again varied considerably, eighteen leaves were involved and the effect was highly significant. Both the afternoon closure and the reduced effect after the first day resemble the preliminary results of Heath and Milthorpe (1950, Fig. 3). This dying away of the rhythm might be expected under continuous illumination but is curious in leaves kept in the dark during the night as in the cases under discussion.

4. *Humidity effects.* The great delay in coming to equilibrium stomatal apertures at very high humidity (Heath and Russell, 1954, p. 8) and the closing effect of low humidity with wheat stomata (p. 272) are in accordance with previous findings (Williams, 1950; Heath, 1950; Heath and Milthorpe, 1950). Williams in the paper cited puts forward the suggestion that if the guard cell walls were more permeable to carbon dioxide under dry conditions this would account for the more rapid closing *and* opening observed. While this would be true of responses to external carbon dioxide, however, and therefore also of indirect light effects operating via the intercellular space carbon dioxide, light responses if mainly due to photosynthetic reduction of carbon dioxide in the guard cells should then be *less* rapid under dry conditions.

It has already been noted (p. 280) that the minimum value of $\log_{10} R_{10,000}$ attained was of the same order as that found by Heath and Milthorpe; the degree of closure even in darkness, however, was much below their maximum for 275 f.c., viz. 0·77 as compared with 1·64. This may probably be attributed to their use of dry air.

5. *The measure of stomatal response.*—The measure used is admittedly an empirical one, but it is difficult to suggest a practicable measure that would be more fundamental. There is nothing fundamental about pore width or area, and even diffusive resistance, though fundamental from the point of view of

stomatal function, has little connexion with their mechanism. The ideal would be turgor difference between guard cells and epidermis,[1] but a method of measuring this has yet to be devised.

The expression used for $\log_{10} R_{10,000}$ (p. 273) involves the following assumptions, also listed by Heath and Milthorpe (1950): (1) That lateral flow through the mesophyll to stomata external to the cup area is negligible. (2) That the rate of air-flow varies directly as the pressure difference. (3) That the conductances of the upper and lower epidermes are in the ratio of the numbers of stomata, i.e. that mean stomatal apertures are at all times the same on both surfaces and that interference between stream-lines flowing through neighbouring stomata is negligible or similar for both surfaces. (4) That the intercellular space resistance normal to the leaf surface is negligible.

The first assumption seems reasonable in view of the fact that α, the ratio of upper to lower stomatal numbers, is actually greater than unity. As pointed out elsewhere (Heath, 1941, pp. 473–4) the greater the value of α the smaller the errors involved in using the measured resistances uncorrected for the effects of the mesophyll and outer stomatal resistances. Milthorpe (unpublished) has made estimates by the method of Penman (Heath, 1941 Appendix), of the effect of such correction in the case of wheat leaves (var. 'Charter') with $\alpha = 1{\cdot}32$ and a somewhat longer and narrower cup than in the present experiments ($4{\cdot}3 \times 0{\cdot}4$ cm.^{2}; washer width 3·5 mm.). These are shown in Table VIII for the maximum and minimum individual resistances found both in the present work and by Heath and Milthorpe (1950).

TABLE VIII[2]

Effects of correction for resistances of mesophyll and stomata outside cup area

	$\log_{10} R_{10,000}$	
	Uncorrected	Corrected
Max. found by Heath and Milthorpe	1·74	1·82
Max. in 'light × CO_2' experiment	0·94	0·96
Min. found by Heath and Milthorpe	2·18	$\bar{2}$·18
Min. in 'light × CO_2' experiment	$\bar{3}$·59	$\bar{3}$·59

It is clear that the correction is in most cases negligible and that practically all the air passes straight through the leaf from the lower to the upper surface except at the very highest resistances.

The second assumption has not been tested for the wheat leaf but was found reasonable for *Pelargonium* (Heath, 1941).

With regard to assumption No. 3 a criticism arises because the leaves were illuminated from above only. In the 'dark' experiment it may be presumed that stomatal apertures were similar on both surfaces, but with increasing illumination the upper stomata may be expected to have opened more widely than the lower, owing to the light intensity being higher especially in the red and blue regions of the spectrum where the leaf pigments absorb most and where stomata show most response (Darwin, 1898; Paetz, 1930; Liebig, 1942).

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[1] The authors are indebted to Professor E. J. Maskell, F.R.S., for this suggestion.

[2] The authors are much indebted to Dr. Milthorpe for providing these figures before publication.

If instead of the equation on p. 273 the following relations could be used

$$R_{\text{observed}} = \frac{R_L}{N_L} + \frac{R_U}{N_U}$$

and

$$R_{10{,}000} = \frac{R_L}{4329} + \frac{R_U}{5671}$$

(where R_L and R_U are the resistances of individual upper and lower stomata respectively), the assumption that $R_L = R_U$ would no longer be made. The above equations show that if the upper stomata open more widely than the lower with increased illumination, the measured resistance R_{observed} will be more and more weighted in favour of the lower stomata. $R_{10{,}000}$ is intended to be an estimate of the combined resistance of 10,000 stomata distributed with a ratio α of 1·31 *and illuminated under the conditions of the experiment*. The equation on p. 273 will thus be satisfactory even when R_L exceeds R_U if the value of α is in fact 1·31, for in this case

$$R_{10{,}000} = R_{\text{observed}} \times \left\{\frac{\text{experimental leaf area}}{\text{area for 10,000 stomata}}\right\}.$$

If α varies much from 1·31 and the difference between R_L and R_U is appreciable, $R_{10{,}000}$ will be overestimated for values of α above 1·31 and underestimated for those below 1·31, but the error seems unlikely to be large. The extremes of α observed in the 'light × CO_2' experiment were 1·48 and 1·02. If assumed values of 2 and 1 be allotted to R_L and R_U, the correct value of $R_{10{,}000}$ is 0·00064. The values estimated using the equation on p. 273 are 0·00065 for α 1·48 and 0·00061 for α 1·02.

Assumption No. 4 is pure assumption but seems reasonable in view of the short path.

6. *Conclusions.* The following possible light effects have been discussed in this and the preceding paper.

1. An indirect effect depending upon the sensitivity of the stomata to carbon dioxide and transmitted by the intercellular space carbon dioxide content as affected by assimilation and respiration in the mesophyll cells. Possible effects of other components of the intercellular space atmosphere have not here been considered.

2. A similar indirect effect transmitted from the mesophyll by carbon dioxide gradients from cell to cell.

3. An indirect effect not operating by reduction of the internal carbon dioxide in the guard cells, transmitted either from the epidermal cells or through them from the mesophyll cells by some agent (chemical or electrical) as yet unknown.

4. A direct effect due to photosynthesis in the guard cells themselves reducing the internal carbon dioxide content.

5. A second direct effect not operating by reduction of the internal carbon dioxide content.

The first effect must occur in Nature and is illustrated by the responses to external carbon dioxide concentration found in 'over', 'through', and 'over-through' types of experiment. The second effect must also in theory operate to some extent, but the relatively long paths concerned suggest that it would be very small. In view of the high ratio of assimilation rate to respiration rate in mesophyll cells, both these effects would be expected to be of the 'all or nothing' type as between dark and light with normal external carbon dioxide (except with the lowest light intensities).

Evidence for the existence of the third indirect effect has been obtained in the apparent 'transmission' of light effects from the experimental to control areas of the 'light' experiment. If accepted this shows that such an indirect effect can be appreciable at considerable distances and from the other side of a 'light barrier'. It is quite unknown whether it originates in the stomata themselves, the other epidermal cells, or the mesophyll.

The results of the experiments described have been interpreted mainly in terms of the fourth and fifth effects (the two direct effects upon the guard cells). This is thought to be the simplest and therefore for the present the most acceptable hypothesis. The evidence for No. 4 (first direct effect) *might*, however, be an artefact due to inadequacy of the rates of 'through' flow, in which case it would really be caused by effect No. 1. The trends attributed to the second direct light effect (No. 5) are probably in part and perhaps entirely due to No. 3; in any case they would appear not to be due to reduction of the internal carbon dioxide content. It thus appears that there are at least two light effects on the guard cells, one operating by reduction of carbon dioxide content (No. 1) and the other not (Nos. 5 or 3). The existence of two others (Nos. 4 and 3 or 5) seems probable.

As far as responses to carbon dioxide are concerned, the finding of Heath and Milthorpe (1950) that wheat stomata in the light show no response to reduction of external carbon dioxide concentration below 0·01 per cent. has received further confirmation, and although the absence of a response can never be proved, it would seem that if it exists it must be of negligible dimensions. In darkness, however, the maximum response is found between zero and 0·01 per cent. external carbon dioxide, and this is attributed to the effects of the carbon dioxide of respiration in the guard cells supplemented by that of the adjacent epidermal cells. The flattenings of the carbon dioxide response curves at high external concentration show that the carbon dioxide operated mechanism can become saturated with carbon dioxide. No information is yet available as to how the carbon dioxide content affects the turgor difference between guard cells and subsidiary cells.

A diurnal rhythm of stomatal movement under constant conditions of illumination and temperature has now been shown to exist for wheat, and since it has previously been found in cherry laurel (Maskell, 1928) and *Pelargonium* (Gregory and Pearse, 1937), it seems probable that it may be of fairly general occurrence.

The authors have great pleasure in thanking Professor E. J. Maskell, F.R.S., for criticism and suggestions in connexion with the interpretation of the results. The junior author held a Post-graduate Studentship from King's College, University of Durham, during the period of the work described.

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The Extension Growth–Time Relationship for *Avena* Coleoptile Sections[1]

T. A. BENNET-CLARK AND N. P. KEFFORD[2]

Botany Department, King's College, University of London

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SUMMARY

1. The growth rates of coleoptile segments supplied with indole-3-acetic acid is not constant with time, but, when the IAA concentration is high, decreases very rapidly.

2. With sufficiently high concentration of IAA, the initial rapid growth may be eventually followed by shrinkage of the tissue.

3. The relation between initial rate of growth and auxin concentration is not significantly different from hyperbolic.

4. The significance of these facts in relation to kinetics of auxin action is discussed.

INTRODUCTION

In a series of papers McRae, Foster, and Bonner (McRae and Bonner, 1952; Foster, McRae, and Bonner, 1952; McRae and Bonner, 1953; McRae, Foster, and Bonner, 1953) have applied enzyme kinetic treatments to the elongations induced in *Avena* coleoptile sections by auxins, alone, and when nteracting with other auxins or with antiauxins, and have drawn conclusions vith considerable bearing upon current theories of auxin action. In this aboratory a study had been made of the interaction of indole-3-acetic acid IAA) with the growth substances of unknown constitution which we have ermed accelerator α and inhibitor β, separated from plant extracts by chromatography (Bennet-Clark and Kefford, 1953; Kefford, 1953). It was of nterest to treat the results kinetically, in the manner of the Californian vorkers, in an effort to learn something of the mechanism of action of these ubstances.

It is probably not unreasonable to assume that the first reaction of auxin s an adsorption or a chemical reaction with an 'active centre' which might be epresented as $C+A \rightleftharpoons CA$. If such an active centre is inside the cell the reaction will be further complicated by diffusion or absorption processes. What s far less clear is that the effective concentration of the complex 'CA' is the actor limiting or controlling extension growth. If with McRae and his o-workers we assume that it is and that no diffusion complications exist, we

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[1] The work described in this paper formed part of a thesis for the degree of Ph.D. in the Jniversity of London.

[2] Address after July 1954: C.S.I.R.O., Division of Plant Industry, Canberra, Australia.

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can calculate a dissociation constant of the complex, comparable to the Michaelis constant of an enzyme. This has a simple form if one can assume a sequence such as

$$C+A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CA \xrightarrow{k_3} C+\text{growth processes}$$

in which k_3 is small compared with k_1 and k_2. This simple relation is evidently claimed to exist by Bonner and co-workers in cases where auxin concentrations are relatively low. At high auxin concentrations they introduce what is apparently claimed as a new kinetic treatment based on a 'two-point attachment' hypothesis. The special case of an enzyme action being inhibited by its substrate is, however, well known and the kinetics of catalase action on hydrogen peroxide was dealt with by Lineweaver and Burk (1934).

They showed that
$$V=\frac{[S]V_{\max}}{[S]+K_s+[S]^n/C}$$

where V and $V_{\max}$ are the initial rate and maximum rate obtained by extrapolation, K_s the Michaelis constant, and $[S]$ substrate concentration, C a constant. A similar relationship was suggested by Foster, McRae, and Bonner, although they did not point out that it had been developed by Lineweaver and Burk, and incidentally does not necessarily demand a two-point attachment of the substrate.

In the treatment both of enzyme kinetics and auxin kinetics it is obviously essential that the rate measured at a given substrate or auxin concentration should be a true initial rate before any effective change in substrate concentration has occurred.

In our study of the kinetics of the effect of the natural inhibitor which we have called β, we have found that the amount of extension growth during a period of 12–20 hours is *not* an estimate of the initial rate of extension. It seemed therefore desirable to reinvestigate the kinetics of the more simple system, where only auxin is present.

The need for care in interpretation of growth rates based on the mean rate occurring during a long time-interval has been pointed out by Audus (1952), who has made measurements at a number of time-intervals in growth studies. Ball (1953), in his work on the growth of *Aegopodium* rhizomes, also pointed out the danger of drawing conclusions on growth from a single measurement made after one time-interval. For their comparison of the growth patterns of IAA and indole-3-acetonitrile (IAN), Bentley and Housley (1953) used measurements of growth at 2-hour intervals.

The work of McRae, Foster, and Bonner mentioned above, to which estimates of initial growth rates and thus measurements of the growth of coleoptile sections with time are fundamental, is reviewed in part by Bonner (1952). McRae and Bonner (1952) suggest that diortho-substituted phenoxyacetic acids and a number of other antiauxins (McRae and Bonner, 1953) are competitive inhibitors of IAA in *Avena* coleoptile section extension. These kinetic studies were done with 5-mm. sections, taken from *Avena* coleoptiles grown at 25–26° for 82–84 hours. The sections were floated on solutions, containing 3 per cent. sucrose, 0·0025 M. potassium maleate buffer (pH 4·5), and the appropriate growth substances, and their extension after 12 hours at 25–26° was taken as a measure of the initial growth rate, since the growth of sections under these conditions was said to be linear with time for 18 hours. The validity of a measurement after 12 hours as an estimate of the true initial growth rate under all conditions is challenged in the present work.

METHODS

The sections of *Avena* coleoptiles used in this investigation were obtained in the following way. Husked and soaked *Avena* seeds ('Victory', from Svalöf) were sown in moist coarse sand in rectangular glass dishes, 12·5 × 12·5 × 4 cm. internal dimensions (sold as half glass-bricks by Pilkington Brothers). They were covered by another half glass-brick and grown in continuous weak red light at 24–25° until 72 hours after soaking when the coleoptiles were 1·5 to 2 cm. long. Coleoptiles of uniform length were harvested and placed 12 at a time in a cutter which removed an apical tip of 3 mm. and took the next 3-mm. section. The sections from each operation of the cutter were distributed amongst the test dishes, which were 3·5 cm. diameter pyrex crystallizing dishes containing 5 ml. of 3 per cent. sucrose solution in glass distilled water of pH 5·0, with the growth substances added where indicated. When an average of 15 sections had been added to the test dishes, these were held at 24–25°. At intervals of between 2 and 4 hours individual test dishes were removed from the environment of 24–25° to one at 20°, where the coleoptile sections were transferred from the test solution to a glass slide and their lengths were measured using a microscope fitted with an eyepiece scale. The sections were then returned to the test solution and the dish returned to 25°. This measuring operation required an average of 3 minutes per dish and did not interfere with the growth of the sections since their growth over 20 hours was not significantly different from that of sections maintained at 25° undisturbed.

The coleoptile section elongation has been expressed as the percentage extension over the original length and the section growth rate as per cent. per hour.

EXPERIMENTAL RESULTS

In the two typical experiments discussed here, the extension growth of *Avena* coleoptile sections, treated in one experiment with 0·0, 0·05, 0·1, 0·2, 0·3, 0·5, 0·7 and 100 mg./l. IAA and in a second experiment with 0·0, 5·0, 10, 20, 30, 40, 50, and 70 mg./l. IAA was measured at intervals of approximately 3 hours for 60 hours. Results are plotted as section extension against time in Figs. 1 *a* and 1 *b* and as section growth rate against time in Figs. 2 *a* and 2 *b* respectively. For clarity the results for all concentrations investigated have not been included; those curves which have been omitted fall in the

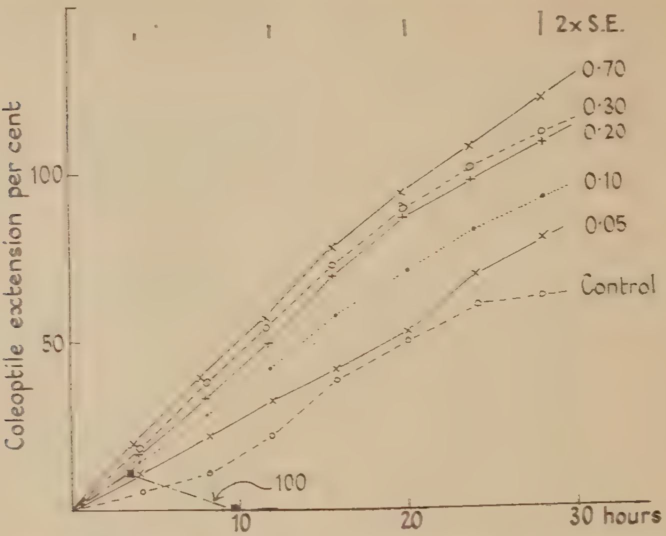

FIG. 1 *a*. Per cent. coleoptile extension with time when treated with auxin concentrations as indicated on the curves. The vertical lines above the curves indicate 2 × standard error. IAA concentrations are given in mg./l.

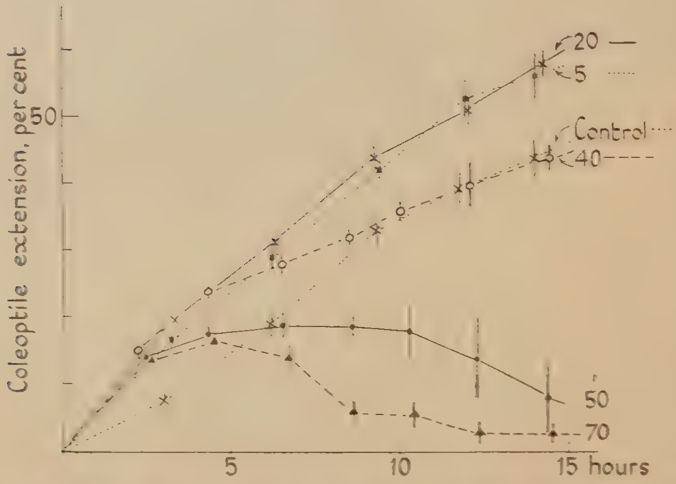

FIG. 1 *b*. A second similar experiment. In this case the vertical lines through the points have a total height of 4 × standard error. Note that the time scale is different from Fig. 1 *a*. IAA concentrations are given in mg./l.

appropriate position between the plotted data. It should be pointed out that these experiments are typical of many that have been carried out with IAA and indole-3-acetonitrile.

Some general results are apparent. The control curves of growth against time (Fig. 1) in both experiment series are slightly sigmoid, giving indications of a 'lag phase' and a point of inflexion (i.e. maximum growth rate) at about 8 hours after the start in one series (Fig. 1 *b*) and 12 hours after the start in the other (Fig. 1 *a*). These sigmoid curves are somewhat apparent with external IAA concentrations up to 0·5 mg./l. The growth rate decreases in the controls and with low external concentrations of IAA from the point of inflexion of the sigmoid curves onwards, probably as a result of the maturing of the extending cells. With higher concentrations of IAA (5–100 mg./l.), however, the

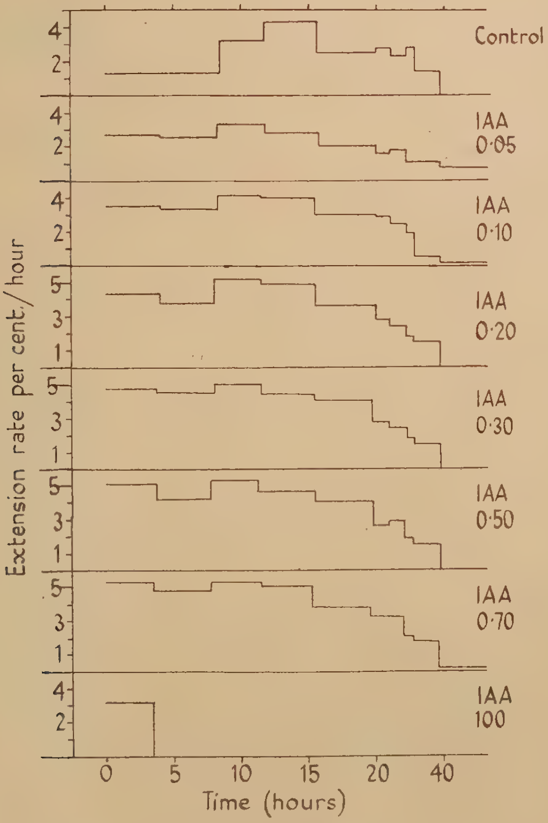

FIG. 2 *a*. Extension rates of coleoptiles against time, based on Expt. 1 *a*. IAA concentrations in mg./l.

decrease in growth rate begins earlier and well before the point of inflexion of the controls; so that, although in concentrations of IAA up to and probably slightly above 0·7 mg. l., growth was reasonably linear with time for about 15 hours, at 5 mg. l. and above, the linear relationship did not hold over any period in the first 15 hours.

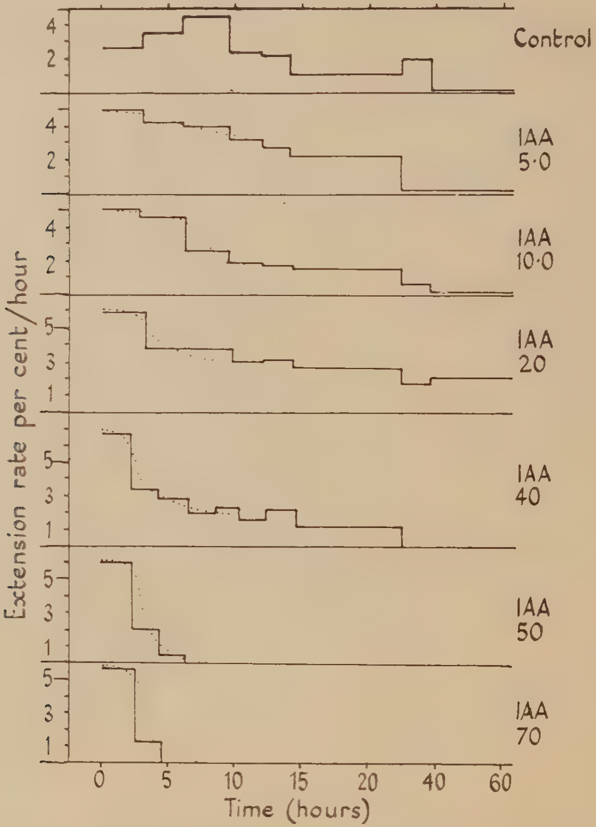

FIG. 2 *b*. Extension rates of coleoptiles against time, based on Expt. 1 *b*. IAA concentrations in mg./l.

It will be noted in Figs. 1 *a* and 1 *b* that with 50, 70, and 100 mg./l. IAA the initial rapid extension growth is followed by a stationary phase and finally by marked shrinkage, which is associated with the ultimate death of the sections. The rapid decrease in growth rate from the high initial rate, especially with high IAA concentrations, suggested that perhaps IAA was converted in tissues into an inhibitor. This view has been put forward by Ball (1953), to explain experimental data on the growth of *Aegopodium* rhizomes, following treatment with IAA solutions. As a check on the presence of any hypothetical inhibitor in the test solution outside the coleoptile sections, the following experiment was done. Coleoptile sections were grown, for 5 and 8 hours respectively, in 50 mg./l. and 30 mg./l. of IAA. The sections were then transferred to fresh solutions of the same compositions. At the same time fresh sections were placed in the original solutions. The growth rates of the original sections are shown by continuous lines in Fig. 3 and the growth rates of the fresh sections in the 'staled' solutions are shown by broken lines. These results show that transfer of the original sections to fresh solutions did not alter the course of their growth and that fresh sections in the 'staled' solutions grew apparently as well as the original sections. Thus the decrease in growth rate in relatively high concentrations of IAA is not due to any production of inhibiting substances which diffuse out into the external solution. It could be due to inhibitors accumulated inside the sections, or to injury to the structure of the tissue or to other secondary reactions of IAA.

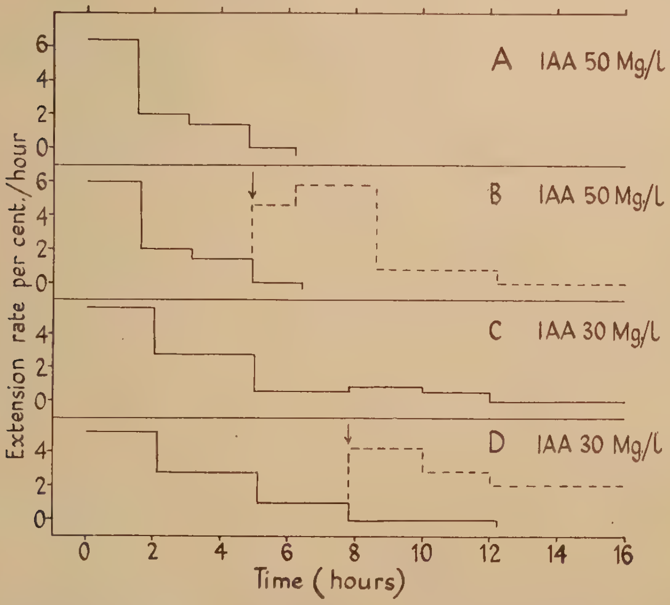

FIG. 3. Coleoptile extension rates against time. A, coleoptiles left in IAA, 50 p.p.m. B, coleoptiles in IAA 50 p.p.m. After the arrow, the broken record represents growth-rate of fresh coleoptiles placed in the staled solution. The continuous record before the arrow indicates the growth rate of these coleoptiles in the IAA solution originally supplied; after the arrow it shows growth rate of the same sections in fresh IAA. C, coleoptiles in IAA 30 p.p.m. D, coleoptiles in IAA 30 p.p.m. After the arrow, broken record represents fresh coleoptiles placed in the staled solution. The continuous record before the arrow indicates the growth rate of these coleoptiles in the IAA solution originally supplied and after the arrow it gives growth rates of original sections in fresh IAA.

DISCUSSION

The extension–time curves for the control sections, which grew in sucrose solution alone, had a slightly sigmoid shape but the shape was not regular. In the experiments recorded here and in others that have been done it was seen that the times, at which the points of inflexion on the sigmoid curves occurred, were spread over a large time range. It is possible that this irregularity is connected with the variation in the physiological age of the coleoptiles from which the sections for the different experiments were taken and that the point of inflexion occurs nearer the start of treatment as the physiological age of the coleoptiles increases. The results of a number of experiments showed that the time from the start of treatment, at which the point of inflexion occurred, increased as the final length to which the sections would grow increased. If the latter is an inverse measure of the physiological age of the coleoptiles, then the possibility is considerable that the point of inflexion in the extension–time curve approaches zero time as the physiological age of the sections increases. If this is the case, then the coleoptiles, used by McRae, Foster, and Bonner in their series of investigations, which were grown at 25–26° for 82–84 hours (i.e. 12 hours longer and at a higher temperature than the coleoptiles used in the present investigation), could have passed the phase of growth which produced the sigmoid effect at the start of the extension–time curve.

McRae and Bonner (1953) found a linear relationship between section growth and time with 1·0 mg. l. of IAA for 18 hours and apparently assumed that the relationship would also be linear at other concentrations. It was shown in Figs. 2*a* and 2*b* that under the present experimental conditions, although the growth–time relationship for IAA at 0·7 mg./l. was roughly linear for 15 hours, it was not linear over this period for 0·05, 0·1 and 0·2 mg./l. Thus the assumption of McRae and Bonner (1953) does not hold here and should not hold under their conditions unless, as suggested above, their sections do not show the sigmoid type of growth.

When disks of some storage tissues are used as experimental material there is a lag phase after cutting in the development of the properties of the disks. A similar effect may be causing the lag phase observed in the growth of the control coleoptile sections. Another possible explanation is a physiological regeneration similar to the regeneration of a physiological tip which occurs in whole decapitated coleoptiles.

The lag phase in the extension-time curves with coleoptiles is, we find, very markedly affected by the osmotic pressure and electrolyte content of the solution bathing the coleoptiles. It is fairly clearly shown when the solutions contain 3 per cent. sucrose, as in the present study and that of McRae *et al.*, but is non-significant in the absence of sugar or with very low concentrations of electrolyte.

A much greater difference between the claims or assumptions of Foster, McRae, and Bonner (1952) and the results presented here is apparent when the extension of sections in high concentrations of IAA is considered. The above authors maintain that, in all concentrations of IAA, 'growth measurements at 12 hours yield the initial growth velocity, since the growth of *Avena* coleoptile sections under the present conditions is linear with time for a period of 18 hours or more'; but, with the experimental conditions used in this study, concentrations of IAA above 5·0 mg./l. gave extension rates that were not constant for any period in the first 12 hours.

As the result of their assumption, Foster, McRae, and Bonner (1952) believe that the initial growth rates of sections in IAA solutions reach an optimum at the concentration, which gives maximum extension in 12 hours. At concentrations above this, the initial rate is supposed by them to fall again. In Figs. 1 *b* and 2 *b* the highest mean growth rate during a 2- to 3-hour initial period is seen to be found with 40 mg./l. IAA, which on the basis of a 12-hour measurement would appear to be well above the optimum concentration for extension. The rates in the initial reading period (Fig. 2*b*) show 50 and 70 mg./l. of IAA to give a lower initial section growth rate than 40 mg./l. and are based on a measurement period of about 3 hours.

The true initial growth rates can only be obtained from the present data by extrapolation to zero hour. Unfortunately it is scarcely possible to effect a truly objective extrapolation.

The rate of extension–time curves at the highest concentrations of IAA *might* be logarithmic so far as the 3 or 4 available points can show. But the corresponding curves for 5, 10, and 20 mg. IAA/l. show something much more like the sigmoid descending portions of the curves obtained at lower IAA concentrations. We are therefore inclined to the view that in all cases the rate of extension–time curves are Ƨ-shaped.

We can therefore produce at least three different types of extrapolation of the rate–time curves to zero hour: sigmoid (which, with these data, is frankly subjective), linear, and logarithmic. The latter two procedures provide estimates of the initial rates much larger than the sigmoid extrapolation which we regard as the most reliable estimate of the true initial rate. The dotted curves in Fig. 2 *b* indicate the sigmoid extrapolation which we believe to be the most reliable estimate.

One can now plot reciprocals of the initial rates against reciprocals of concentrations of IAA. As one approaches zero reciprocal concentration, one should in fairness plot both reciprocal initial rates obtained by sigmoid extrapolation and those obtained by log or linear extrapolation. The data have been plotted in Fig. 4. Using the initial rates derived by sigmoid extrapolation one obtains the points shown as X and Δ. The X points are obtained from low concentration data and the Δ points are derived from high auxin concentrations and the latter are subject to largish standard errors together with the uncertainty of extrapolation. There is little reason to suppose that they significantly diverge from the straight line drawn through the remaining points.

In order to show the high concentration data more clearly, these same Δ points are plotted as o points with the abscissa scale multiplied by 100. The continuous line *AC* has the same slope as *AB* and shows fairly clearly the extent of divergence of initial rates from the hyperbola on which *AB* and *AC* are based. It would of course be possible to fit these high concentration data to a different hyperbola which would yield the broken line when reciprocals are plotted, but we have no reason to think that divergence from the line *AB* is significant.

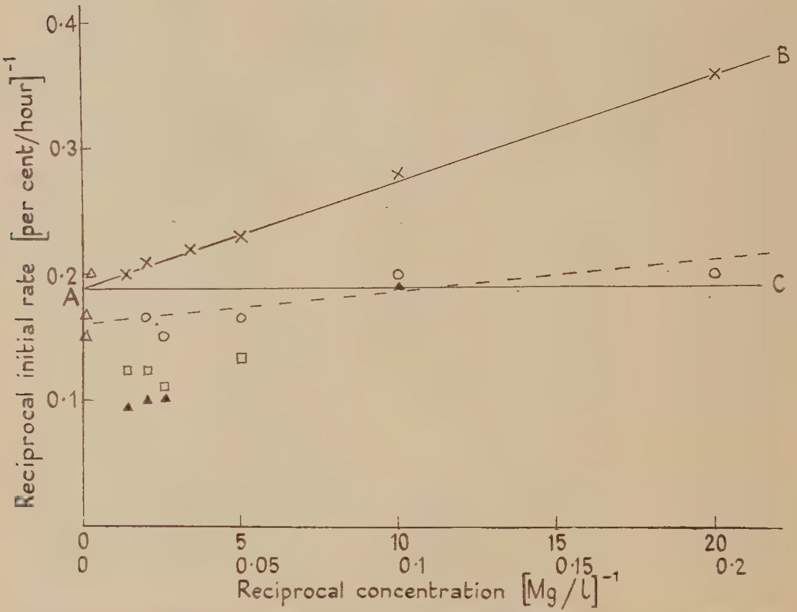

FIG. 4. Reciprocal initial rates plotted against reciprocal concentrations of IAA. Points shown as X are derived from Exp. 1 *a*, △ from Exp. 1 *b*.; the upper abscissa scale refers to these points. The same points are replotted as o with an abscissa scale 100 times larger to indicate distribution of initial rates at very high concentrations. The line *AB* is redrawn as *AC* for this increased abscissa scale, shown as the lower scale. The points △ and o are derived from a sigmoid extrapolation. Linear extrapolation would yield points shown as □, exponential extrapolation as ▲.

If one used linear or exponential extrapolation instead of sigmoid, the reciprocal initial rates would be as shown by □ points and ▲ points respectively. These diverge significantly from the hyperbola which yields *AC* on plotting reciprocals.

One could treat the data given in Figs. 1 *a* and 1 *b* in the same manner as has been done by Foster, McRae, and Bonner. The slopes of the lines joining the origin and the points showing the lengths of coleoptiles after 10 hours' exposure provide 'rates' the reciprocals of which are plotted in Fig. 5 (continuous curve, points X). This is essentially the same type as that of the lower half of Fig. 2, p. 1016, in the paper by Foster *et al.* (1952). When one uses the slopes obtaining during the first 2 hours one gets the broken curve and points (o).

The continuous curve of our Fig. 5 like that of Foster *et al.* is clearly an experimental artefact.

In our data of reciprocal initial rates, plotted against reciprocal concentrations of auxin, it can be readily seen that there is no justification for the view that the inverse initial rates at high concentrations do not fall on the same straight line as those for lower concentrations. In other words, the rate–concentration relationship is hyperbolic over the whole concentration range.

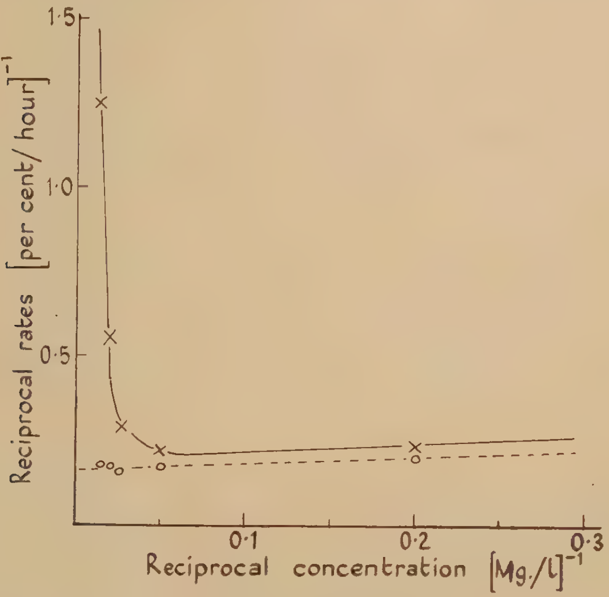

FIG. 5. Reciprocal rates plotted against reciprocal concentrations of IAA. The record ——×—— gives reciprocal rates based on the mean rate during the first 10 hours of extension growth. The record - - - o - - - represents corresponding values based on the mean rate during the first 3 hours.

The intercept in the reciprocal plot enables one to evaluate $V_{\max}$ and from the slope one can obtain a parameter that could be interpreted as a dissociation constant. It is, however, in any case still a matter for debate whether enzyme kinetic treatment of this type is applicable to multi-enzyme systems of the degree of complexity that must be involved in growth.

The cause of the rapid decrease in rate of extension with time at higher IAA concentrations is still uncertain. The data of Ball have suggested that IAA may be converted into an inhibitor, possibly the substance β. There is, however, clear evidence that, if so, it accumulates in the tissue rather than in the external solution (see p. 299 and Fig. 3).

Originally it was intended to study the effectiveness of β as an inhibitor of IAA in extension growth and it was hoped that the kinetics would enable one to deduce whether this inhibition was a form of competitive inhibition or not. Stocks of β were used up at an early phase of the study before it was realized that the growth–time relationship was so far from linear. In fact, in presence of β the initial rate of extension due to IAA is increased, but the total extension occurring in a 12 to 20 hour period is considerably reduced.

It is clear therefore that the growth effects evoked by materials eluted from chromatograms must be studied not by mere observations of extension at single limited times but by study of extended rate–time curves.

In conclusion, one might consider how Foster, McRae, and Bonner came to think that the mean 12-hour extension rate equalled the initial rate. The rate–time curve is, we find (unpublished work by Mr. H. M. Hurst), markedly affected by low concentrations of salts such as potassium chloride or potassium maleate which Bonner and co-workers employed as a buffer. Adequate buffering of the IAA when employed at 10^{-6} to 10^{-8} M. is likely to be achieved at the protoplasmic surface, so in the present study the marked complication introduced by K^+ or Na^+ in a buffer was avoided. In the presence of ions, the initial rapid extension rates brought about by auxins are considerably reduced and may thus have passed unnoticed by the Californian workers.

ACKNOWLEDGEMENTS

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The Isolation of Tyramine from a West African *Crinum* Species

L. FOWDEN

Department of Botany, University College, Gower St., London, W.C.1

AND

J. DONE

Human Nutrition Research Unit, Medical Research Council Laboratories, Holly Hill,
Hampstead, London, N.W.3

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SUMMARY

Tyramine, a rarely encountered constituent of higher plants, has been isolated from the exudates obtained from the cut flower-stalks of *Crinum yuccaeflorum* Salisb. It was present at a concentration of 7 mg. per ml. and represented at least 90 per cent. of the amino nitrogen present in the exudate. The significance of this observation is briefly discussed. The distribution of tyramine and amino-acids in the leaf, root, and bulb tissues of the plant was also investigated.

INTRODUCTION

FROM the literature tyramine would appear to be a constituent of only a limited number of plant species. It was first described by Barger (1909) as a constituent of the ergot obtained from the parasitic fungus, *Claviceps purpurea*, present on rye. Crawford and Watanabe (1914, 1916) isolated tyramine from several species of American mistletoe (*Phoradendron flavescens*, *P. californicum*, and *P. villosum*), and Ostenberg (1915) also reported it in the European mistletoe, *Viscum album*. It has also been isolated from *Capsella bursa-pastoris* (Shepherd's purse) by Boruttau and Cappenberg (1921), and from the seeds of *Silybum marianum* (milk thistle) by Ullmann (1922). Recently Erspamer and Falconieri (1952) reported that tyramine was present in barley germs.

During a survey of different plants for the possible presence of γ-methyleneglutamine and γ-methyleneglutamic acid (isolated from groundnut plants, Done and Fowden, 1952), a strong reddish-purple ninhydrin reactive spot was found on chromatograms prepared from the exudates obtained from the cut flower-stalks of *Crinum* (see experimental section for further details of species). This spot had an R_F value of 0·95 in phenol-ammonia, and therefore resembled an amine rather than an amino-acid (see Bremner and Kenten, 1951). The area containing the spot also gave the Pauli reaction when treated with diazotized sulphanilic acid as described by Dent (1948). The unknown substance was also strongly absorbed from aqueous solutions by charcoal. Since the foregoing properties are shown by histamine and tyramine, the

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unknown substance was compared with these amines and found to be inseparable from tyramine on chromatograms developed in five different solvent systems.

The unknown substance was obtained in pure form from the pooled exudates of many individual plants by a procedure designed to isolate a phenolic amine. Melting-point determinations, ultra-violet absorption spectra, and X-ray powder photography showed that the pure substance isolated (or prepared derivatives) was identical in all respects to tyramine (or the corresponding derivatives).

Tyramine constituted almost all of the ninhydrin reactive material present in the exudates, although traces of aspartic acid, glutamic acid, and other amino compounds were detected. The distribution of tyramine and of amino-acids in the leaf, root, and bulb tissues of the plant was also examined, but no indication of the function or mode of synthesis of tyramine has been obtained.

EXPERIMENTAL

Plant material. Although at the present time some doubts exist concerning the exact application of species names to plants of the genus *Crinum*, the species used here appeared identical to that described as *Crinum yuccaeflorum* Salisb. by Hutchinson and Dalziel (1936). The plants were growing under natural field conditions in Gambia, West Africa. A limited number of young plants have also been grown under greenhouse conditions in England.

Chromatographic techniques. Paper chromatograms were prepared by methods essentially those of Consden, Gordon, and Martin (1944). The solvents used were redistilled phenol saturated with 0·3 per cent. (w/v) ammonia solution (referred to as phenol-NH_3 hereafter), the *n*-butanol-acetic acid mixture of Partridge (1948), the collidine-lutidine mixture of Dent (1948), 7 per cent. (v/v) *n*-propanol, and water-saturated *n*-butanol. 0·1 per cent. (w/v ninhydrin in water-saturated *n*-butanol was used in the normal manner as the spraying agent. Two-dimensional chromatograms were run in phenol-NH_3 followed by butanol-acetic acid as solvents.

Isolation of the unknown substance from exudates. At the time of flowering the flower-heads were removed and the exudates from the cut stalks were collected and pooled. 75 ml. of exudate were obtained. The amount of exudate obtained from single plants varied greatly, but was almost 1 ml. in some instances. Using butanol-acetic acid as the developing solvent, chromatographic examination of the exudates from many individual plants revealed the same ninhydrin reactive spots, the unknown spot always being predominant. It was also noticed that the exudate obtained from a cut made across the perianth tube or style of an individual flower (the flower-head had three to eight flowers arranged as an umbel) contained the same spots, whilst the exudate resulting from a cut across the basal portion of a leaf again contained the unknown, together with increased amounts of several amino-acids.

300 ml. absolute ethanol were added to the 75-ml. exudate and the precipi

tated proteins were removed by filtration and discarded. The filtrate was shaken with 3 volumes of chloroform to remove the ethanol (see Awapara, 1948), when 65 ml. of an aqueous phase separated. After the addition of a further 100 ml. of chloroform to the organic layer, it was extracted with 25 ml. of water, giving a second aqueous layer (35 ml.), which was combined with the first. 25 g. of activated charcoal prepared as described by Partridge (1949) was added to this aqueous phase, and the mixture stirred for 90 minutes. Saunders, Lackner, and Schochet (1931) demonstrated that tyramine and other alkaloidal bases were strongly absorbed by charcoal. The charcoal was then removed by filtration and washed with distilled water, the filtrate being discarded. The unknown substance, which had been absorbed by the charcoal, was eluted by shaking the residue with 100 ml. of 25 per cent. (w/v) acetic acid solution containing 5 g. of A.R. phenol. The charcoal was removed by filtration and discarded, and the filtrate twice extracted with 100 ml. of ether to remove the phenol. The remaining aqueous layer was evaporated to dryness *in vacuo* to remove the acetic acid. A reddish-brown coloured residue remained (0·7 g.).

The residue was dissolved in 20 ml. of distilled water and made alkaline by the addition of a slight excess of solid sodium carbonate. The solution was then extracted six times with 10-ml. portions of water-saturated *n*-butanol. Most of the colouring matter remained in the aqueous phase at this stage. The pooled butanol extracts were then evaporated to dryness at a temperature of less than 50°. The residue was twice extracted with 30 ml. of boiling xylene and the dark coloured gummy residue discarded. The xylene extract was concentrated to about 25 ml. and, on cooling in ice, glistening crystals of the unknown substance separated out. They were twice recrystallized from xylene and finally obtained as white plate-like crystals (yield, 100 mg.).

Characterization as tyramine. The unknown material isolated was shown to be identical with tyramine purified from a sample of commercial tyramine hydrochloride. The following techniques were used.

Melting-point determinations: the sample melted sharply at 160·5°, tyramine at 161·5°, and a mixture of the sample with tyramine at 160·5°. Picrates of the sample and tyramine were prepared and melted as follows: sample 201°, tyramine 202°, and mixed melting-point 201°. The sample and tyramine were also treated by the Schotten-Baumann procedure, using excess benzoyl chloride in order to benzoylate both the —NH_2 and phenolic —OH groups. The dibenzoyl derivatives were twice recrystallized from 70 per cent. ethanol and melted as follows: sample 167°, tyramine 168°, mixed melting-point 167·5°. All melting-points recorded are uncorrected.

Provided the derivative was recrystallized more often, it was possible to prepare pure dibenzoyltyramine by direct treatment of the exudate with benzoyl chloride in the presence of 2 N. sodium hydroxide.

Elementary analysis: the nitrogen content of the sample was 10·1 per cent. (duplicate micro-Kjeldahl) ; tyramine ($C_8H_{11}ON$) requires 10·2 per cent. nitrogen. The elementary analysis of the dibenzoyl derivative of the sample agreed with that of dibenzoyltyramine (Found: C, 70·1; H, 5·43; N, 4·08 per cent. $C_{22}H_{19}O_3N$ requires C, 70·7; H, 5·23; N, 4·07 per cent.). The analyses were performed by Drs. Weiler and Strauss, Oxford.

Chromatographic analysis: the sample had R_F values identical with those of tyramine when the two substances were run on one-dimensional chromatograms developed with each of the five solvents listed earlier. It was also inseparable from tyramine when a mixture of the two substances was chromatographed. On two-way chromatograms the sample moved to a position close to that occupied by phenylalanine and the leucines.

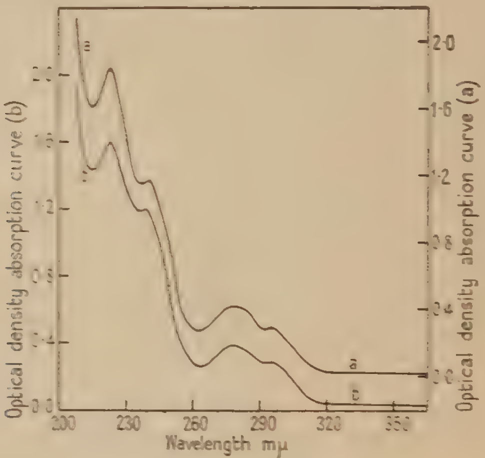

FIG. 1. The absorption of ultra-violet light of wave-lengths 200 to 360 mμ by equimolar aqueous solutions of (*a*) tyramine, and (*b*) sample.

Colour tests: in addition to giving the Pauli reaction when chromatograms were treated with diazotized sulphanilic acid, the sample also gave an intense red coloration when treated with Millon's reagent. It also gave a strongly positive xanthoprotein test when boiled with dilute nitric acid, followed by addition of excess ammonia solution.

Ultra-violet absorption spectra: the absorption of ultra-violet light of wave-lengths 200 to 300 mμ by dilute equimolar aqueous solutions of the sample and tyramine were determined using a Unicam S.P. 500 spectrophotometer. The spectra are shown in Fig. 1. Strong absorption peaks were shown by both substances at wave-lengths of 223 and 280 mμ, whilst subsidiary peaks were found to occur at 240 and 290 mμ.

X-ray powder photographs: the dibenzoyl derivatives of the sample and of tyramine were shown to be identical by X-ray powder photography. Fig. 2 is a reproduction of the diffraction patterns obtained. The derivatives were four times recrystallized from 70 per cent. ethanol under identical conditions. In an earlier X-ray comparison of chemically pure derivatives of the sample and tyramine, recrystallized without strict control of the ethanol concentration and cooling conditions, the diffraction patterns obtained from the two substances were not identical. Although all diffraction lines given by the sample exactly coincided with similar lines shown by the tyramine, the relative intensities of the lines varied for the two substances. This difference may have been caused by the derivatives existing in two distinct crystalline forms, the relative proportions of which varied for the two preparations made under slightly different conditions.

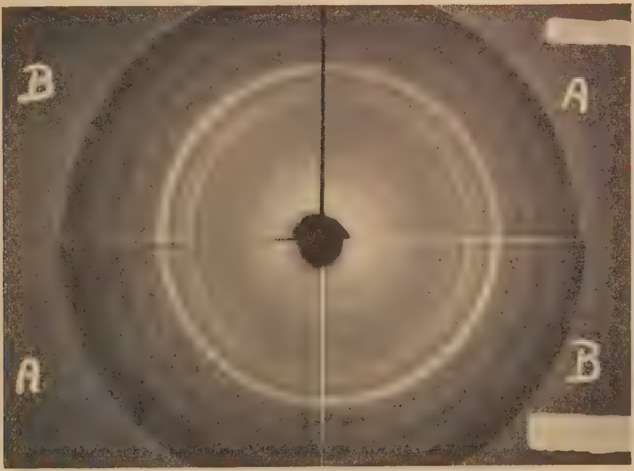

FIG. 2. A reproduction of an X-ray powder photograph showing the identical nature of the dibenzoyl derivatives of the sample (A) and tyramine (B).

Distribution of tyramine within the plant. The amount of tyramine in the combined exudates was determined after separation from other ninhydrin reactive substances on one-dimensional chromatograms run in butanol-acetic acid by the method of Fowden (1951). The tyramine constituted over 90 per cent. of the ninhydrin reactive material in the exudate, and was present at a concentration of 7 mg./ml.

The distribution of tyramine in the greenhouse-grown plants was studied by means of two-dimensional chromatograms. Root, leaf, and bulb material were separately harvested and the fresh tissues ground with 80 per cent. ethanol (5 ml. for each g. of plant material) and a little washed sand. The extractions were continued for 24 hours with shaking. After centrifuging each sample the clear supernatants were concentrated *in vacuo* and aliquots applied to the chromatograms. A semi-quantitative assay of the relative amounts of tyramine and amino-acids present in the extracts was made by comparing the areas and colour intensities of the ninhydrin-developed spots present on the chromatograms. Table I presents the results obtained. The relative concentrations of each substance are indicated, using an arbitrary scale of 0 to 5 units; 0 indicates that a substance was not detected in the concentrated extracts.

Table I

Showing the relative concentrations of ninhydrin reactive compounds in 80 per cent. ethanol extracts of fresh root, bulb, and leaf material

	Root extract	Bulb extract	Leaf extract
Asparagine	5	4	1
Glutamine	3	5	2
Aspartic acid	3	3	3
Glutamic acid	4	3	5
Alanine	4	2	4
Tyramine	3	2	1
Serine	1	1	1
Glycine	2	2	1
Threonine	2	1	1
Valine	0	1	0
Leucines	0	1	0
Basic amino-acids	2	2	2
γ-aminobutyric acid	1	0	1

DISCUSSION

Chromatograms of freshly collected exudate showed, in addition to tyramine, spots of aspartic acid and glutamic acid, and small quantities of an additional ninhydrin-reactive substance moving as a small well-defined spot flowing just in advance of tyramine when butanol-acetic acid was used as solvent. This substance gave the Pauli reaction, and was perhaps closely related to tyramine. Erspamer *et al.* (1952) showed that N-methyl-tyramine flowed slightly faster than tyramine in the solvent systems they used, and that, like tyramine, it could be detected using the Pauli reaction. The substance present in the pooled *Crinum* exudates was apparently unstable and gradually disappeared during storage in the refrigerator.

The most striking feature of the present investigation is undoubtedly the fact that tyramine, a compound rarely encountered in higher plants, should be present in an exudate from cut flower-stalks of the *Crinum* species examined to the virtual exclusion of all other ninhydrin-reactive compounds. This is contrary to our experience of exudates from a number of other plant species, where several amino compounds have been present in significant concentrations together with smaller amounts of similar compounds. A comparable finding can be cited, however, in the exudate obtained from groundnut plants, where γ-methyleneglutamine was the only amino compound present in measurable amounts (Done *et al.*, 1952). The volume of the exudate from a single *Crinum* stalk was far greater than that which could have been produced by damage to the cells in the immediate regions of the cut. This is supported by the fact that if pressure was applied to the cut stalks in order to increase the volume of exudate obtained, the exudate then contained larger amounts of amino-acids (resulting from a greatly increased number of damaged cells). Groundnut-stem exudates showed a similar behaviour when pressure was applied.

Large volumes of extremely dilute guttation fluids can be exuded from cut vine-stems, under certain favourable conditions. Such dilute solutions probably arise from the xylem elements of the stems. The exudates from *Crinum* were, however, relatively concentrated solutions, presumably largely derived from the phloem. It must therefore be assumed that in part the exudate represented the fluids being translocated along the stem, and that tyramine was the principal form in which organic nitrogen was conveyed to the developing flower-head. There the tyramine may simply accumulate or be converted to other alkaloidal bases such as hordenine, N.N-dimethyl-tyramine, although the literature indicates that hordenine is confined to plants of the Gramineae and Cactaceae. Alternatively the nitrogen of tyramine may be available via some unknown mechanism for new protein synthesis at the growing-points.

It seemed possible that tyramine may be produced within the plant by a mechanism involving the decarboxylation of tyrosine, but no supporting evidence has been obtained. Tyrosine was added to extracts of fresh root, bulb, and leaf material made by grinding the tissues with a minimum volume of pH 5·6 phosphate buffer and a little washed sand, but no detectable formation of tyramine occurred in a 24-hour incubation period at 25°. The apparent absence of such a decarboxylation mechanism is perhaps not surprising in view of the finding that tyrosine was not detectable in extracts of these tissues (see Table I).

Although tyramine was present in the exudate at concentrations greatly in excess of those of any other amino compound, Table I indicates that this position is reversed in extracts of each of the plant organs examined. Tyramine was present in root, bulb, and leaf material, but aspartic acid, glutamic acid, alanine, asparagine, and glutamine were always present in greater amounts. Roots contained more tyramine in relation to the total ninhydrin-reactive material than did the bulbs, which in turn contained more than the leaves. The amides, glutamine, and asparagine, were predominant in the bulb (storage organs), and this distribution can be compared with the similar situation in potato tubers (Thompson and Steward, 1951). The distribution of the amino-acids in *Crinum* leaves is similar to that found in the leaves of other plants, where alanine, aspartic acid, and glutamic acid have usually been shown to be predominant.

Aspartic acid, glutamic acid, and their corresponding amides are thought to play a central role in the nitrogen metabolism of higher plants, although the experimental evidence supporting this contention has been derived from studies on relatively few plant species. It may be that when other species are eventually examined, alternative metabolic pathways will suggest themselves. In the case of *Crinum*, a metabolism involving tyramine may be superimposed on the more usual metabolic reactions involving the dicarboxylic amino acids and their amides.

We are greatly indebted to Sir Edward J. Salisbury, F.R.S., Director, and to Mr. Sealy of the Royal Botanic Gardens, Kew, for assistance in identifying the plant species used in this work, and to Professor Kathleen Lonsdale, F.R.S., for X-ray powder photographs. We would also like to acknowledge the receipt of grants in aid of apparatus from the Royal Society.

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The Exchangeability of Potassium and Bromide Ions in Cells of Red Beetroot Tissue

J. F. SUTCLIFFE
Department of Botany, King's College, London

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SUMMARY

The exchangeability of potassium and bromide ions accumulated by cells of red beetroot tissue has been examined under various experimental conditions by means of radioactive tracers.

It is established that the cells contain a certain amount of easily exchanged ions which is largely independent of the salt content of the tissue. The remainder of the salt does not exchange appreciably within 24 hours either during absorption or when accumulation is stopped by low temperature, potassium cyanide, high internal salt content, or by an insufficient preliminary washing of the material.

An hypothesis is proposed that the easily exchanged ions are distributed throughout the intercellular spaces, cell walls, and in parts of the protoplasm, whilst those which do not readily exchange may be situated in the cell vacuoles, or else strongly associated with protoplasmic constituents. The results suggest that a considerable barrier to the free diffusion and exchange of ions is located in the region of the tonoplast of a plant cell, and the metabolic transport of ions across this membrane is discussed.

An examination of the changes which occur in the amounts of readily exchanged potassium during the washing of freshly cut beet disks in aerated distilled water indicates that the protoplasm probably acquires a capacity to fix ions more strongly as a result of this treatment. This supports the view that an effect of washing on the capacity of cells to absorb salts metabolically is to increase the number of 'absorption centres' involved.

INTRODUCTION

THERE is now a considerable amount of evidence (Lundegårdh, 1940; Robertson and Turner, 1945; Machlis, 1944) supporting the view that energy required for ion accumulation is derived from a cyanide-sensitive, salt-stimulated component of respiration at least in some plant tissues. On the basis of experiments with excised wheat roots, Lundegårdh has proposed that cytochromes are the carriers of anions into plants cells, and a mechanism of salts absorption involving these substances has been suggested (Lundegårdh, 1946). A difficulty in the acceptance of this hypothesis is the observation that the rates of ion uptake and salt respiration are not always directly related. Robertson and Wilkins (1948) showed, for example, that the maximum rate of salt absorption by carrot tissue is attained when the ion concentration of the medium in which the cells are suspended is much higher than that which causes the maximum stimulation of respiration. Moreover, it appears that

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with red beet cells the rate of potassium accumulation decreases with increasing internal salt concentration although the level of cyanide-sensitive respiration remains unaffected (Sutcliffe, 1952).

In order to reconcile this latter observation with the Lundegårdh hypothesis it was suggested that as the internal concentration of ions in the cell vacuoles increases there may be an increase in the rate of outward movement of potassium from the tissue, so that net absorption is decreased although the rate of gross uptake remains constant, as is in fact indicated by the level of salt respiration. The possibility that ion accumulation in cells represents the balance between active uptake and a passive leakage has already been proposed by Krogh (1946) and by Lundegårdh (1947). Some evidence against the hypothesis that the rate of uptake of potassium by beet cells is controlled in this way was obtained (Sutcliffe, 1952) by determining the amount of leakage which occurred from the tissue when metabolic absorption was prevented by the presence of potassium cyanide. It may, however, be argued that the inhibitor can also influence outward movement if this directly or indirectly depends upon metabolism. In order to get more unequivocal data about the transfer of ions in both directions through the protoplasts of beet cells, a series of experiments was designed with radioactive isotopes, ^{42}K and ^{82}Br. Our earlier investigation, which was concerned only with potassium absorption, has been extended to include the uptake of an anion, since, in view of the suggestion of Lundegårdh that cations enter plant cells along an electrical gradient created by the metabolic accumulation of anions, it seems likely that the resistance offered by the protoplasts to diffusion and exchange of the two ion species might be rather different.

A determination of the rates of movement of ions in both directions through plant protoplasts may be of interest in another connexion. If the Lundegårdh mechanism is employed exclusively by cells, it is clear that the ratio of anions or cations absorbed to oxygen uptake in salt respiration cannot exceed 4 (Robertson and Wilkins, 1948). For wheat roots Lundegårdh (1950) observed much lower ratios than this (0·4–0·7), but with carrot tissue Robertson and Wilkins were able to show that the theoretical value is approached but not exceeded under optimum conditions. These data were based on determinations of the net amount of ion uptake, so it is possible that, if there is an appreciable outward movement of salt from the tissue, gross absorption may exceed net accumulation and the ratio be greater than 4. In that event it is clear that the Lundegårdh hypothesis must be supplemented by an alternative mechanism. This is the proposal made by Davies and Ogston (1950) in connexion with the secretion of hydrochloric acid by gastric mucosae of the frog, since they observed ratios of HCl-secreted oxygen-involved as high as 11.

The exchangeability of ions in various plant and animal tissues has already been determined by other investigators, and a perusal of the literature indicates that the rate as well as the total amount of exchange depends very much on the experimental material and the particular ion studied. A number of experiments with animal cells have shown that ions in some tissues will exchange completely with those of the medium and the rate of turnover may be high. Krebs, Eggleston, and Terner (1951) calculated, for example, that 3–4 per cent. of the total potassium in brain slices was exchanged per minute at 40° C., and an even higher rate (7–10 per cent.) was recorded for retina cells under the same conditions. Sheppard, Martin, and Beyl (1951) have established that in mature canine erythrocytes there is a rapidly exchanging fraction of the total potassium content, whilst the remainder exchanges only slowly. It was shown that the rate of exchange of potassium by human blood-cells is not increased by an increase in the amount of plasma potassium, but it is closely correlated with the intracellular concentration of this ion.

The position with regard to plant tissues appears similarly to be quite complex. Brooks (1940) has shown that sodium and potassium ions from the external medium exchange completely with those occurring in the protoplasm of *Nitella* within 15 seconds, but ions in the vacuole do not exchange appreciably even in several hours. Broyer and Overstreet (1940) found that about 10 per cent. of the potassium ions in barley roots are exchanged readily and that the rest are relatively non-exchangeable. Experiments with strontium and iodine, on the other hand (Jacobson and Overstreet, 1947), showed that there is no appreciable fraction of these elements present in a non-exchangeable form since 85 per cent. of the total strontium ions exchanged in 1 hour and 70 per cent. of the iodide in 4 hours.

In the present investigation the rates of exchange of potassium and bromide ions by beet cells has been studied with 'salt-saturated' as well as with 'low-salt' material; in the presence of potassium cyanide at a concentration which inhibits metabolic uptake and salt respiration, and at two different temperatures, one of which (25° C.) permits the rapid accumulation of ions, and the other (5° C.) prevents it. Finally, a study has been made of the changes in exchangeability of potassium which occur during the washing of freshly cut tissue in aerated distilled water in an attempt to correlate this with changes in the absorptive capacity of the cells which are observed as the result of this treatment.

MATERIALS AND METHODS

The experimental material consisted of disks of red beetroot tissue (*Beta vulgaris*, var. 'Crimson Globe') prepared as has been described in a previous paper (Sutcliffe, 1952), except that in the present investigation thinner disks (0·5 mm. rather than 0·75 mm. thick) were employed. In most of the experiments the disks were used after they had been washed in aerated distilled water for about 7 days at 25° C., during which time a maximum absorptive capacity was developed. When a 'salt-saturated' tissue was required the disks were washed for the same time and under the same conditions in 0·02 M. potassium bromide instead of distilled water as the preliminary treatment.

Absorption experiments were then conducted on samples of 30 disks (0·7–

0·8 g. fresh weight) placed in 4-ml. aliquots of the culture medium so that the same volume ratio of tissue medium was maintained as in our previous investigations. The exchange rates for potassium and bromide ions were determined in separate experiments with 0·02 M. potassium bromide labelled with either ^{42}K or ^{82}Br as the external solution. The medium was prepared by dissolving the radioactive material (either K_2CO_3 or NH_4Br) in distilled water to a suitable dilution and making this up to the required concentration with inactive potassium bromide. The amounts of HCO_3^- and NH_4^+ ions introduced into the final media by this procedure were negligible in comparison with the amounts of potassium and bromide present.

Determinations of radioactivity were made at intervals during an experiment on 3-ml. aliquots of the medium diluted to 10 ml. with distilled water by means of a G.M. liquid counter tube and an N.R.D. ratemeter. The radioactivity of the experimental solutions was compared directly with that of a control medium which was not in contact with the tissue to remove errors introduced through the decay of the isotope during the period of the experiment. The initial radioactivity of the medium was in each case adjusted so that after dilution to 10 ml. the 3 ml. of experimental solution gave a count of about 400 per second in the ^{42}K experiments and about 200 counts per second in the experiments with ^{82}Br. A preliminary investigation showed that beet cells under the conditions employed here can withstand radiation from the isotopes of at least 100 times this intensity for 24 hours without the rate of uptake of either oxygen or ions being affected. A similar insensitivity of cell permeability to radiation injury has been reported by Mullins (1939) working with *Nitella* coenocytes.

After the radioactivity determinations had been made the solutions were allowed to stand for several days until it was judged that the isotopes were sufficiently decayed for them to be handled without risk. Each 10 ml. of solution was then diluted to 50 ml. and analysed for potassium and bromide. Potassium was determined by flame photometry, and bromide by titration with silver nitrate using ammonium chromate as indicator.

The data obtained from the radioactivity and total uptake determinations are expressed below as μ-equivalents of ions absorbed per gram fresh weight of the tissue. In an experiment three replicate flasks were set up and analysed for each treatment, and, as each observation was repeated twice, the results presented represent the mean of six determinations on separate cultures. Replications of a treatment on the same day generally varied by less than 5 per cent., and, in separate experiments on different days with the same batch of material, agreement was usually better than 10 per cent. In most of the experiments described below determinations of the total potassium and bromide content, as well as the radioactivity, were made on the material at the end of an investigation as a check for the uptake measurements based on analysis of the external medium. This was accomplished by boiling each sample of 30 disks separately with its residual 1 ml. of medium (after removal of the 3 ml. aliquot), and 10 ml. of distilled water at 100° C. for 30 minutes.

10 ml. aliquots of these solutions were then analysed for potassium bromide and radioactivity by the methods described above. From these determinations the uptake of ions was calculated and compared with that which was indicated by analysis of the external medium. Generally the figures agreed to within 15 per cent., and when they differed by more than 20 per cent. the whole experiment was repeated.

EXPERIMENTAL RESULTS

(*a*) *Exchangeability of ions in well-washed, low-salt tissue.*

The uptake of potassium and bromide ions by well-washed low-salt beet disks from a 0·02 M. solution of potassium bromide at 25° C. during 6, 12, 18, and 24 hours, determined both by the change in radioactivity of the medium and by direct analysis, is shown by the data of Table I.

TABLE I

K^+ *and* Br^- *absorbed (μ-equiv./g. fresh weight) by well-washed beet disks from 0·02 M.* KBr *at 25° C. determined by (A) radioactivity measurements, (B) direct analysis of the external medium*

	K^+		Br^-	
Time (hours)	(*A*)	(*B*)	(*A*)	(*B*)
6	41·5	39·5	23·8	25·5
12	55·3	58·5	40·5	42·3
18	73·3	72·0	57·8	56·5
24	82·0	83·3	68·5	68·3

The results of Table I show that the uptake of both potassium and bromide ions determined by the change in radioactivity of the external solution is not significantly different from that which is indicated by direct analysis. This means that the decrease in activity of the medium during absorption can be accounted for by the change in potassium or bromide content, and exchange of ^{42}K or ^{82}Br for inactive ions already present in the tissue appears to be a relatively unimportant contributory cause.

In the case of bromide this result is not unexpected, since the initial halogen content of the material was found to be small (less than 5 μ-equiv./g. fresh weight), and it is possible that bromide will not exchange easily with other anions which may be present. On the other hand, however, the beet disks contained initially about 60 μ-equivalents of potassium per gramme of fresh weight, and about 15 μ-equivalents of sodium with which potassium ions from the external solution might exchange through a suitably permeable membrane. 4 ml. of 0·02 M. potassium bromide solution contain 80 μ-equivalents of potassium, and 30 disks have initially about 45 μ-equivalents inside, so that complete exchange would reduce the radioactivity of the external medium by one-third even if the possible exchange of potassium for sodium is neglected. About one-tenth of the total exchange should have been readily detected in the present experiments, and clearly the potassium has not exchanged even to this extent within 24 hours.

A second series of experiments was designed to determine the effect of reduced metabolic activity on the rates of potassium and bromide exchange with washed tissue. Metabolism was inhibited in one case by low temperature (5° C.) which reduces the rate of oxygen uptake by beet disks to about 5 per cent. of the rate observed at 25° C., and in another by the presence of KCN at a concentration of 0·001 M., which inhibits salt respiration without noticeably influencing ground respiration (Sutcliffe, 1952). The results of these experiments are presented in Table II.

TABLE II

K^+ *and* Br *absorbed* (*μ-equiv./g. fresh weight*) *by well-washed beet disks from 00·2 M.* KBr, (i) *at 25° C.*, (ii) *at 5° C.*, (iii) *at 25° C. in the presence of 0·001 M.* KCN. *Other details as for Table I*

	Temp. (°C.)	KCN	Time (hours)	K^+ (*A*)	K^+ (*B*)	Br^- (*A*)	Br^- (*B*)
(i)	25	—	6	38·5	38·3	21·8	22·5
	25	—	12	58·0	56·8	38·3	39·0
	25	—	18	74·8	70·3	56·3	54·8
	25	—	24	85·3	81·5	63·5	61·0
(ii)	5	—	6	16·8	15·5	5·5	5·8
	5	—	12	17·5	16·3	6·3	6·5
	5	—	18	18·0	17·5	7·0	7·3
	5	—	24	18·3	17·3	7·3	7·5
(iii)	25	+	6	17·5	16·8	6·5	7·1
	25	+	12	18·8	18·0	8·0	7·5
	25	+	18	19·3	18·8	8·8	8·3
	25	+	24	19·8	19·3	8·5	9·0

The data of Table II show that when the rate of metabolism is reduced either by low temperature or by the presence of potassium cyanide, the absorption of potassium and bromide ions is depressed. Nevertheless a considerable uptake of both ions occurs during the first 6 hours which in the case of potassium is equal to nearly a half of the ions absorbed at 25° C. in the absence of the inhibitor. Considering bromide, on the other hand, uptake in 6 hours is reduced to about 25 per cent. by the low temperature or treatment with cyanide. Absorption under these conditions must be attributed mainly to non-metabolic processes such as diffusion, exchange, or adsorption. The close agreement between the uptake determined by radioactivity measurements and by direct analysis suggests that ion exchange is limited although the small deviations observed in the potassium series both at low temperature and in the presence of potassium cyanide may be attributed to this cause. Most of the ions absorbed by the tissue during this treatment probably pass by diffusion into the intercellular spaces and cell walls, whilst some are adsorbed on cell-wall and protoplasmic constituents. On account of the low rate of metabolism it is unlikely that an appreciable amount of salt is transported into the vacuoles during the first 6 hours of the experiment.

The results of Table II also show that after 6 hours there is a relatively slow absorption of ions which may occur actively through the incomplete inhibition of metabolic processes under the experimental conditions employed. There is no evidence from the data that exchange of either potassium or bromide ions takes place after 6 hours, and it is certain that at the end of 24 hours most of the potassium originally present in the tissue remained unexchanged.

(*b*) *Exchangeability of ions in well-washed, high-salt tissue.*

Although it appears therefore that there is no appreciable exchange of potassium or bromide ions between beet cells and the external medium with well-washed tissue, even when metabolism is inhibited, the possibility remains that diffusion outwards of ions plays an important part in controlling the rate of absorption of salts by salt-saturated material, and this will now be considered. High-salt disks were prepared as has been described above, and transferred in batches of 30 to 4 ml. aliquots of 0·02 M. KBr. containing ^{42}K or ^{82}Br. The changes in radioactivity and ion content of the external medium determined after 6 and 24 hours at either 5° C. or 25° C. are shown by the data of Table III.

TABLE III

K^+ *and* Br^- *absorbed* (*μ-equiv./g. fresh weight*) *by salt-saturated beet disks from a solution of* 00·2 *M.* KBr, (i) *at* 25° *C.*, (ii) *at* 5° *C.*, (iii) *at* 25° *C. in the presence of* 0·001 *M.* KCN. *Other details as for Table I*

				K^+		Br^-	
	Temp. (°C.)	KCN	Time (hours)	(*A*)	(*B*)	(*A*)	(*B*)
(i)	25	—	6	10·3	0·5	1·8	0·3
	25	—	24	11·0	0·8	2·3	0·5
(ii)	5	—	6	10·8	0·3	2·0	0·5
	5	—	24	11·8	0·8	2·5	0·3
(iii)	25	+	6	11·3	0·8	2·8	—
	25	+	24	11·5	1·3	2·8	0·8

The results of Table III show that although there was only a small net uptake of potassium or bromide ions during the course of the experiment there was an appreciable exchange of ions between the medium and the tissue. The amount of this exchange was not significantly affected by temperature and was only a little greater on the whole after 24 hours than after 6 hours. An analysis of the material after an experiment showed that the salt-saturated disks contained about 242 μ-equiv./g. fresh weight of potassium, and about 170 μ-equiv./g. fresh weight of bromide. It appears therefore that 4–5 per cent. of the total potassium, and 1–2 per cent. of the total bromide in the tissue was readily exchanged at either the high or the low temperature in 6 hours, and the remainder did not exchange appreciably during 24 hours. The data of Table III show further that the exchangeability of potassium and bromide ions in beet cells is unaffected by the presence of potassium cyanide at a concentration of 0·001 M.

Confirmation of the observation that a large proportion of the potassium and bromide ions accumulated in the high-salt tissue do not exchange within 24 hours at either 5° C. or 25° C. was obtained by another experiment in which disks which had first been washed for 7 days in distilled water were next allowed to absorb salt for 24 hours at 25° C. from a 0·02 M. KBr solution containing ^{42}K or ^{82}Br. The change in radioactivity of the medium during this time was determined and the tissue was then transferred to several changes of distilled water, and subsequently to an inactive KBr solution, or to distilled water for 6 or 24 hours at 5° C. or 25° C. The radioactivity of the medium was then measured and the results obtained are presented in Table IV. A correction has been applied to the figures to allow for decay of the isotope during the experimental period, and background counts were subtracted.

TABLE IV

Radioactivity (counts per minute) of the external medium after 6 and 24 hours when disks containing ^{42}K *or* ^{82}Br *were transferred to either 0·02 M.* KBr *solution or to distilled water at 5° C. or 25° C.*

		K^+		Br^-	
Temp. (°C.)	Time (hours)	H_2O	KBr	H_2O	KBr
5	6	28	128	13	33
5	24	35	135	16	30
25	6	32	107	11	41
25	24	23	91	8	28

The results shown in Table IV demonstrate that only a few radioactive ions appear in the external medium when distilled water alone is present, and suggest that the rate of diffusion of potassium or bromide ions from the tissue under the conditions of the experiment is rather slow. The detection of more intense radioactivity in the KBr medium may be attributed to a limited exchange of ions between the solution and the disks which appears to be completed within 6 hours. From the determinations of the potassium and bromide content and radioactivity of the material before transference to the inactive medium it was calculated that the amounts of ions exchanged represented about 6 per cent. of the total in the case of potassium and 2 per cent. for bromide.

(*c*) *Changes in exchangeability of potassium during washing*

The experiments described above have all involved the use of tissue in which the absorptive capacity is assumed to be fully developed following washing either in distilled water or a mineral salts solution for 7 days. An investigation was next undertaken of the changes in exchangeability of potassium which occur during the washing treatment, as a capacity to absorb is developed. Freshly cut disks were placed in distilled water at 25° C. for varying periods of time up to 8 days, and then transferred in batches of 30 to 4-ml. aliquots of 0·02 M. KCl at 5° C. for 4 hours. The amount of potassium absorbed during this time was then determined by an analysis of the external medium with the flame photometer. Next the tissue was placed from the inactive solution to another 0·02 M. potassium chloride medium containing ^{42}K, and the amount of potassium exchanged in 6 hours at 5° C. was estimated by measurement of the changes in radioactivity and total potassium content of the medium. Finally, the total amount of potassium in the disks immediately before transferring to potassium chloride was determined on replicate batches of tissue. The results of this experiment are presented in Table V.

The data of column *A* of Table V show that there was a small increase in the potassium concentration in the medium when disks were transferred to it immediately after cutting, which was probably the result of leakage of ions from cut or damaged cells. After the tissue had been washed in aerated distilled water for 2 days at 25° C. it absorbed about 4·8 μ-equiv./g. fresh weight of potassium upon transfer to the salt solution and the amount of uptake increased when the time of washing was prolonged up to 8 days. The results in column *B* of Table V shows that the amount of easily exchangeable potassium in the tissue after it had been placed in potassium chloride for 4 hours at the low temperature decreased from about 16·2 μ-equiv./g. fresh weight in freshly cut tissue to about 10·7 μ-equiv./g. fresh weight for disks which had been washed for 8 days before transferring to the salt solution. It may be noted that the decrease in amount of exchangeable potassium cannot be attributed to a reduction in the total amount of salt in the tissue, since the amount of potassium which is easily exchanged continued to decrease for 6–8 days although the total potassium in the disks does not change significantly after about 2 days of washing.

TABLE V

Amounts of K$^+$ *(μ-equiv./g. fresh weight), (A) absorbed in 4 hours at 5° C., (B) subsequently exchanged in 6 hours at 5° C., (C) present initially in the tissue, with disks which were washed in aerated distilled water for various lengths of time and transferred to 0·02 M.* KCl

Days of washing	Uptake (*A*)	(*B*)	(*C*)
0	−1·3	16·2	68·0
2	4·8	15·1	52·0
4	10·1	12·1	50·9
6	17·6	10·9	50·6
8	18·4	10·7	50·7

A comparison of the data of Tables III and V shows that the amount of readily exchanged potassium is not much different in well-washed low-salt disks treated for a few hours with potassium chloride at low temperature, and salt-saturated tissue, although the total potassium content in the two cases is quite different.

DISCUSSION

The results presented above indicate that potassium and bromide ions do not readily diffuse out of beet cells even when the latter are in the 'salt-saturated' condition, and it is evident therefore that the limitation in the rate of absorption which occurs when the internal solute content of the tissue is high cannot be attributed to this cause but must rather be due to a decrease in the rate of gross uptake. Since it has been shown (Sutcliffe, 1952) that the level of 'salt respiration' is not significantly affected by the amount of salt in the tissue, it is clear that with our material there is no constant relationship between this component of respiration and the metabolic uptake of ions. Whereas the calculated ratio of cations absorbed O_2 involved is about 3 during the first 6 hours in the experiments with beet, the ratio is reduced to less than 2 when the calculation is made over 24 hours. Lundegårdh (1950) has suggested that the low ratios observed with wheat roots may be attributed to the utilization of a part of 'anion respiration' for the transport of 'native ions' in tissue and to leakage of ions from the cut surfaces of the excised roots. It seems unlikely that, in the present case, salt saturation causes a considerable increase in the amount of organic anions and leakage is negligible, so the conclusion appears inevitable that the level of salt respiration is determined by effects of the salt on the respiratory system in the plant cell irrespective of the number of ions actually transported from the medium.

Nevertheless, in view of the relationship swhich have been observed between inhibition of salt respiration and absorption by cyanide by Lundegårdh and others (Machlis, 1944; Robertson and Turner, 1945; Sutcliffe, 1952) it seems likely that accumulation and a cyanide-sensitive component of respiration are closely linked together. It is probable that the energy required for salt absorption, as well as for other metabolic processes in cells, is derived from cytochrome-mediated respiration in those organisms where cytochrome is an important terminal oxidase system. When other terminal oxidases are involved ion accumulation may be related to different respiratory components (Osterlind, 1951; Russell, 1953). When cytochrome oxidase is concerned it seems probable that the ion absorption mechanism is more flexible than Lundegårdh suggests, and it is unlikely therefore that cytochrome can itself be the carrier for anions.

Lundegårdh (1946) has suggested that in plant roots it is the anions which are actively transported whilst cations move passively across the protoplasts along the electrical gradient so created. On the other hand, for some animal cells the active movement of cations rather than anions has been proposed (e.g. Solomon, 1952). If either cations or anions move passively through the protoplasm into the vacuole of a plant cell, then those inside will be exchangeable with others present in the external medium. The evidence presented above shows that neither potassium nor bromide ions exchange readily, and it appears that protoplasts present a considerable barrier across which neither anions nor cations can be transported except by the direct intervention of a metabolically controlled mechanism. An attractive hypothesis to account for ion absorption is that proposed by Jacobson, Overstreet, King, and Handley (1950). They have suggested that ions react with metabolically produced carrier substances in the protoplasm to form complexes which subsequently break down to release free ions according to the equations: $HR+M^+ \rightleftharpoons MR+H^+$, $ROH+A^- \rightleftharpoons RA+OH^-$.

Another hypothesis postulating the intervention of separate carrier substances for anions and cations is that of the redox pump (Conway, 1953). Alternatively both ions may be bound to an amphoteric nitrogen compound which can escort them together across the plasma membrane to the place at which breakdown takes place (Steward and Street, 1947).

The data presented above show that ions can be bound into plant cells with various degrees of exchangeability. Similar observations have been made by Jacobson and Overstreet (1947) with barley roots, and by Olsen (1948) studying the potassium content of beech leaves. That the state of the potassium fixed in beet disks may be influenced by the extent of the preliminary washing treatment is shown by the data of Table V. It is improbable that ions absorbed in 4 hours by the tissue at low temperature penetrate into the vacuoles since Brooks (1940) and Hoagland and Broyer (1942) found that, although ions enter the protoplasm of *Nitella* coenocytes readily, movement into the vacuole occurs more slowly, and is probably prevented entirely when metabolism is inhibited. The potassium absorbed by beet disks in 4 hours at low temperature may therefore be located mainly in the intercellular spaces, cell walls, or protoplasts. Since the volume of the intercellular spaces and cell walls does not increase after about 2 days washing of the disks (Sutcliffe, 1952), it is evident that the increased uptake of salt with more prolonged washing must be attributed to an increased affinity of the protoplasm for ions. This may be associated either with an overall synthesis of more protein molecules to which the ions are adsorbed, or formation of specific protoplasmic constituents capable of combining with potassium.

The results of Table V show that, following a more prolonged period of washing, the potassium absorbed on transference to potassium chloride solution is less easily exchanged. Of about 18·4 μ-equiv./g. fresh weight of potassium ions absorbed non-metabolically with well-washed disks only about 10·7 μ-equiv. subsequently exchanged under the conditions of the experiment. It is probable that those ions which were readily exchanged were located in the cell walls, intercellular spaces, or loosely bound in the protoplasm, whilst those which did not exchange were probably more firmly attached to protoplasmic constituents or already in the vacuoles. Jacobson and Overstreet (1947) have indicated that ions may be bound in protoplasm by the formation of chelated compounds from which exchange at a measurable rate does not occur, and various other possibilities have been suggested by Conway (1953). It is possibly in forms such as this that the potassium becomes increasingly bound following washing, owing to the synthesis of suitable acceptors during this treatment. A part of the non-exchangeable potassium may be associated with mitochondria, since the accumulation of ions in these structures has been demonstrated by Bartley and Davies (1952). Again, however, the amount absorbed in mitochondria as in vacuoles may be relatively small if metabolic energy is required for its accumulation.

The relatively large amount of potassium which becomes fixed in a non-exchangeable form at the low temperature with well-washed tissue (7·7 μ-equiv./g. fresh weight) suggests that the absorptive centres may be located throughout the protoplasm rather than only at the surface of it, as was suggested by Lundegårdh (1946). The proposal that protoplasts contain a large number of negatively charged regions to which cations may be fixed has already been made by Blinks (1940). Our observation indicates that the main barrier to diffusion and exchange in a plant cell may be situated in the region of the tonoplast separating protoplasm from the vacuole. Other evidence supporting this conclusion has been derived from determinations of the 'apparent free space' with roots (Hope and Stevens, 1952; Butler, 1953). This hypothesis is also in agreement with the observation of Bennet-Clark and Bexon (1943) that metabolically active substances such as organic acids are relatively inert when they are confined to cell vacuoles.

Since the amount of potassium fixed in the protoplasm of beet cells in a non-exchangeable form is increased as a result of washing the tissue in distilled water, it is possible that this is related to the enhanced capacity of the tissue to absorb ions metabolically following this treatment. It may be suggested that a part of the potassium absorbed into the protoplasm at low temperature may be combined with newly synthesized or reactivated carrier molecules which appear during washing and are responsible for the increased rate of metabolic absorption of which the cells are capable when placed under suitable conditions. This implies that the potassium carrier complex may be formed without the utilization of metabolic energy, and the association may be one of physical adsorption or exchange for hydrogen as is suggested by Jacobson and Overstreet (1947). Subsequently the complex must be metabolized until it finally breaks down to release free ions into the vacuoles. The mechanism outlined by Conway (1953, Scheme II) is one of the ways in which this may be achieved. When the free energy gradient becomes too great through increase of internal concentration of salt, the breakdown of the ion carrier complex may be prevented, and free carrier become unavailable for the acceptance of more ions from the external medium. In this manner a reduction in the rate of absorption as internal concentration is increased may be achieved without invoking the existence of outward leakage.

Spatial transport of the ions across the tonoplast may occur either by turning over of the carrier molecule from one side of the membrane to the other

(Lundegårdh, 1946), by association of the carrier with movable particles such as mitochondria (Robertson, 1951), or contraction of protein molecules may be involved (Goldacre, 1952).

In conclusion therefore it may be proposed that the transport of ions into the vacuoles of plant cells occurs by a metabolic mechanism involving specific carrier substances by means of which movement across a highly impermeable tonoplast is achieved. Failure of the tissue in the present experiments to absorb ions either through insufficient washing or because of salt-saturation is attributable to the absence of carrier molecules in a suitable condition. Whereas in the first case the carrier molecules may be absent or perhaps inactivated through the presence of an inhibitor (Skelding and Rees, 1952), in the second they are probably present in a combined form with ions and therefore ineffective. When absorption is prevented in well-washed, low-salt tissue by low temperature or potassium cyanide it is possible that association of ions with the carrier can take place but uptake is limited since subsequent breakdown of the complex and reformation of carrier molecules does not take place because of the lack of metabolic energy.

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A Study of the Absorption and Utilization of Phosphate by Young Barley Plants

III. THE RELATIONSHIP BETWEEN THE EXTERNAL CONCENTRATION AND THE ABSORPTION OF PHOSPHATE

R. SCOTT RUSSELL, R. P. MARTIN, AND O. N. BISHOP

Department of Agriculture, University of Oxford

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SUMMARY

The absorption of phosphate by young barley plants was studied using a series of solutions ranging in concentration from 0·0003 to over 30 p.p.m. P. The duration of most of the experiments was 24 hours. The relationship between the external concentration and absorption was found to be sigmoid, the slope of the curve being maximal when the external concentration was in the range of 0·1 to 0·3 p.p.m. P. It was concluded that the reduction in the slope of the curve, when the external concentration was reduced below 0·1 p.p.m. P was due to an increasing fraction of the phosphate which entered the plants being retained in metabolic systems in the root, from which it was subsequently released and returned to the outer medium.

These results indicate that when the external concentration was lowered below 0·1 p.p.m. P the plants absorbed progressively decreasing fractions of the nutrient available to them. The significance of this from the viewpoint of the growth of plants under the condition of phosphate shortage is discussed.

INTRODUCTION

In the preceding papers of this series (Russell and Martin, 1953; Russell, Martin, and Bishop, 1953) the partition of labelled phosphate between roots and shoots was examined in plants supplied for short periods with concentrations of labelled phosphate ranging from 0·000316 to 31·6 p.p.m. P. The effect of the amount of phosphate entering the plant on the extent to which recently absorbed phosphate was subsequently lost to the outer medium was also studied. The importance was stressed of distinguishing between two active processes which are responsible respectively for the metabolic retention of phosphate in roots and for the transference of phosphate into the stele. The present paper is concerned with a further stage in the analysis of the effect of external concentration on the phosphate nutrition of intact plants, namely the relationship between the external concentration and the rate of absorption of phosphate.

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EXPERIMENTAL METHODS

Barley plants at the second-leaf stage, which had been raised in water culture without external sources of phosphate, but in the presence of a balanced supply of other nutrients, were treated with varying concentrations of labelled phosphate for periods which, in all but one experiment, did not exceed 24 hours. Concentrations of labelled phosphate ranging from 0·000316 to 31·6 p.p.m. P were used. A full description of the methods employed has been given elsewhere (Russell and Martin, 1953). It is, however, necessary to note three problems inherent in the experimental procedure:

(i) Significant losses of phosphate occurred from the more dilute solutions due to adsorption on to the walls of glass culture vessels. When the initial concentration of the external medium was more than 0·1 p.p.m. P, losses due to this cause were not significant during 24 hours; when concentrations of 0·001 p.p.m. P were employed, 25 per cent. or more of the phosphate was lost.

(ii) The external solutions were not replenished during the course of experiments, and were depleted to a considerable extent except at the higher concentrations of phosphate.

(iii) Significant amounts of the phosphate in the more dilute solutions were not in true solution, but in a form in which they could be removed by centrifuging.

The possibility of treating glass surfaces to reduce adsorption losses was examined; Rubin (1949) claimed that silicones were effective in this respect, while Hall and Mackenzie (1948) recommended butylmethacrylate for treating glass vessels and dimethyldichlorsilane (a silicone) for treating pipettes. A silicone (D.C. 200) and methylmethacrylate lacquer, substances similar to those used by Hall and Mackenzie, were employed to coat the walls of culture vessels. Non-wetting surfaces were produced, but no significant reduction occurred in adsorption from dilute phosphate solutions. Equally unsatisfactory results were obtained with 'cetavlon' (cetyltrimethylammonium bromide) which also produces a non-wetting surface on glass (Norrish and Russell, 1947); furthermore, this substance significantly retarded the growth of plants. In view of the negative nature of these results, no further experiments were undertaken on this question. It may, however, be noted that since these investigations were completed Jackson and Garrard (1953) have stated that unplasticized polystyrene is effective in reducing adsorption. Other workers have found paraffin-wax to be of value, but it could not be used conveniently in the present work.

The complications resulting from the adsorption of phosphate on the walls of culture vessels were reduced by the fact that at the pH employed (5·5–6·5) the adsorption is virtually irreversible; no appreciable amount of adsorbed phosphate returned into solution when phosphate-free solutions of the same pH were placed in vessels on the walls of which phosphate had previously been adsorbed (Russell and Martin, 1953). Hence the concentration of phosphate in solution at any instant was a valid measure of the phosphate available or adsorption by plants. As will be shown later, the fact that the external nedium was depleted by two mechanisms, namely absorption by plants and dsorption on the walls of culture vessels, did not prevent an assessment of the general relationship between the external concentration and absorption.

The fact that significant amounts of phosphate could be removed from dilute solutions by centrifuging gave rise to a complication less easy of definition. Separate experiments were therefore undertaken to examine the behaviour of phosphate in such solutions. It is convenient to defer consideration of this subject until the main experimental data have been considered.

EXPERIMENTAL RESULTS

The effect of variations in the external concentration of phosphate on the rate of absorption by plants

In six experiments the effects of variations in the external concentration on the extent of phosphate absorption were examined. Details of the treatments employed are summarized in Table I; other results from Expts. A, B, and E were discussed in the first paper of this series (Russell and Martin, 1953). Since 24 hours was the experimental period employed in the majority of experiments, the effects on absorption during this period will first be considered.

Table I

Summary of experimental treatments

Experiment	A	B	E	Q	R
Vol. of culture solution (ml.)	300	300	300	600	300
No. of replicates .	4	5	5	6	5
Duration of treatment (hours)	24	1, 3, 6, and 24	24	24	48
Concentrations of labelled phosphate p.p.m. P	31·6	31·6	—	—	—
	10·0	—	10·0*	10·0	—
	3·16	—	—	—	—
	1·00	1·00	—	1·00	—
	0·316	—	—	—	0·316
	0·100	—	0·100*	0·100	—
	0·0316	0·0316	—	—	0·0316
	0·0100	—	—	0·0100	—
	0·00316	—	—	—	0·00316
	0·00100	0·00100	0·00100*	0·00100	—
	0·000316	—	—	—	—

* In Expt. E three levels of ferric chloride (2·0, 0·2, and 0·02 p.p.m. Fe) were combined factorially with potassium dihydrogen phosphate. In the other experiments no salts other than potassium dihydrogen phosphate were used.

On account of the wide range of concentrations, it is impracticable to express the relationship between external concentration and absorption on a linear scale as a continuous curve. It has, however, been possible to present the results without distortion of slope by drawing the curve in sections t different scales. In Fig. 1 the results of Expt. A are shown in this manne Curve I covers the range of initial concentrations 0·00316–0·01 p.p.m. F curve II, 0·01–0·316 p.p.m. P; curve III, 0·316–10 p.p.m. P; curve IV (inset

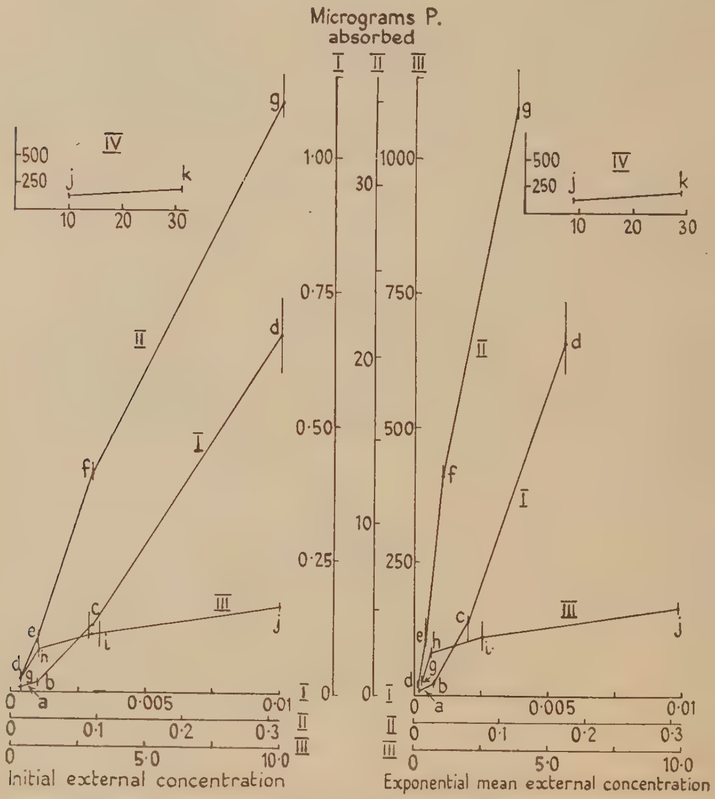

FIG. 1. *Experiment A*: Relationship between external concentration and the absorptio of phosphate by barley plants during 24 hours. Vertical lines indicate the range of values each point. The external concentration, in p.p.m. P, is expressed both as the initial and ex ponential mean values. Each graph is presented in four sections to different scales (see text Key to treatments (initial concentrations, p.p.m. P): *a*, 0·000316; *b*, 0·00100; *c*, 0·0031 *d*, 0·0100; *e*, 0·0316; *f*, 0·100; *g*, 0·316; *h*, 1·00; *i*, 3·16; *j*, 10·0; *k*, 31·6.

10–31·6 p.p.m. P. The range of the four replicate samples at each concentra tion is shown, and the external concentration is expressed both as the initi value and as the exponential mean. In Fig. 2 lines of unit length parallel t those joining each two adjacent points in Fig. 1 have been connected to giv continuous curves. This diagrammatic representation is not to scale, bu changes in the slope of the curves are more conveniently shown than in Fig.

The relationship between external concentration and absorption is sigmoid, and the inclination of the curve relative to the horizontal axis is maximal between the initial concentrations of 0·1 to 0·03 p.p.m. P. While the general form of the curve is unaffected by the basis on which the external concentration is expressed, the concavity of the lower part of the curve is most marked when the external concentration is expressed as the exponential mean. Since this measure of concentration reduces the errors which depletion may cause in the measurement of absorption, the sigmoid form of the curve cannot be dismissed as a consequence of the varying degrees to which the solutions were depleted.

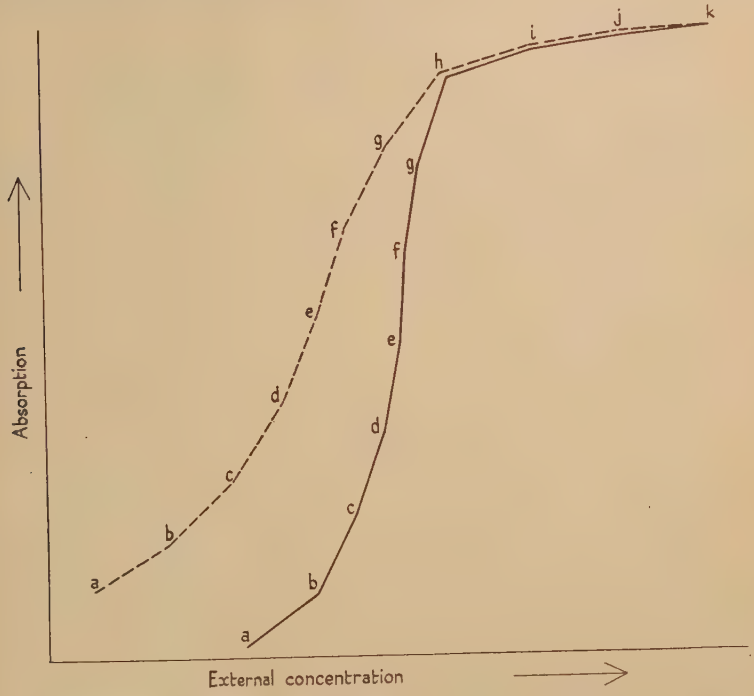

FIG. 2. *Experiment A*: Diagram showing changes in slope of relationship between external concentration and absorption. Co-ordinates not to scale (see text). Solid line: external concentration expressed as initial value. Broken line: external concentration expressed as exponential mean. Key to treatment as in Fig. 1.

It is generally accepted that absorption is proportional to external concentration when the external solution is sufficiently dilute, while in more concentrated solutions the rate of absorption increases less rapidly than the external concentration and tends towards a maximal value. Interest therefore attaches to the concavity of the lower part of the curves shown in Figs. 1 and 2, which indicate that when the external concentration was raised from very low levels, absorption increased in more than linear proportion to external concentration over a considerable range (i.e. up to initial concentrations of 0·1 p.p.m. P). Attention in the present investigation was therefore confined to the factors which determine the form of this part of the absorption curve.

To facilitate the examination of this question, consideration was given to the validity of expressing absorption in relative terms according to the equation

$$\text{Relative absorption} = \frac{\text{Phosphate absorbed} \times 100}{\text{Phosphate content of the solution}}.$$

A similar measure was used by Stiles and Skelding (1940), who, however, employed ratios and not percentages. If relative absorption were examined at three widely spaced points, e.g. 10, 0·1, and 0·001 p.p.m. P, and the highest value occurred at the intermediate concentration, the sigmoid nature of the curve would be established.

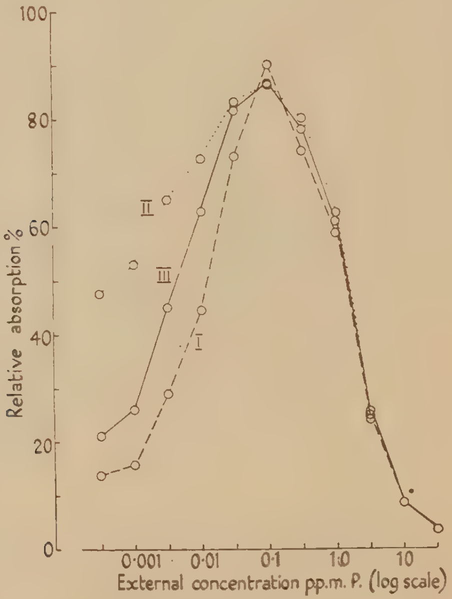

FIG. 3. *Experiment A*: Relative absorption calculated by formulae *i*, *ii*, and *iii* (see text).

To test the validity of this transformation the results of Expt. A have been converted into terms of relative absorption. Since the initial (N) and final (F) content of the culture medium and the amount of phosphate absorbed by the plants (P) were determined, relative absorption could be calculated in three ways, since it is equal to: (*i*), P/N; (*ii*), $(N-F)/N$; or (*iii*). $P/(P+F)$.

Ideally these three expressions should give identical values since N should equal the sum of P and F. Losses of phosphate on to the walls of culture vessels, however, cause different values to be obtained by the three methods when dilute solutions are employed. It is therefore important to consider the effect of adsorption losses on the validity of estimates of relative absorption. In Fig. 3 values for Expt. A calculated by the three methods are compared.

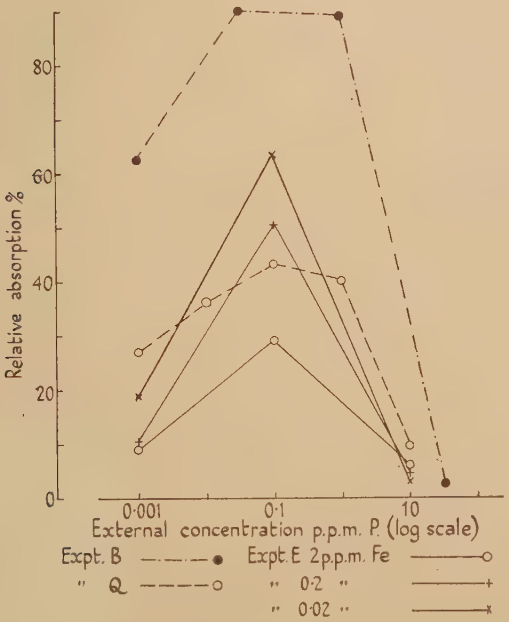

FIG. 4. Relative absorption in experiments of 24 hours duration.

Formula *i* has clearly underestimated relative absorption from dilute solutions, since it makes no allowance for the fact that adsorption losses during the course of the experiment will have reduced the amount of phosphate available to plants treated with dilute solutions; formula *ii* has conversely overestimated relative absorption from such solutions since phosphate lost by adsorption on to the walls of the vessels will contribute to the estimated values for relative absorption. The correct values must, therefore, lie between those derived by formulae *i* and *ii*. Both formulae, however, show well-marked maxima at the initial external concentration of 0·1 p.p.m. P and thus indicate that the absorption curve is sigmoid. A closer approximation to the true values for relative absorption is given by formula *iii*, which in effect ignores phosphate lost by adsorption on to the walls of the culture vessels. Fig. 3 shows that values calculated by this formula fall between those derived by formulae *i* and *ii*. Clearly, however, the values obtained by formula *iii* will be strictly correct only if adsorption losses occurred instantaneously at the beginning of the experiment and preceded absorption by the plants. None the less, this

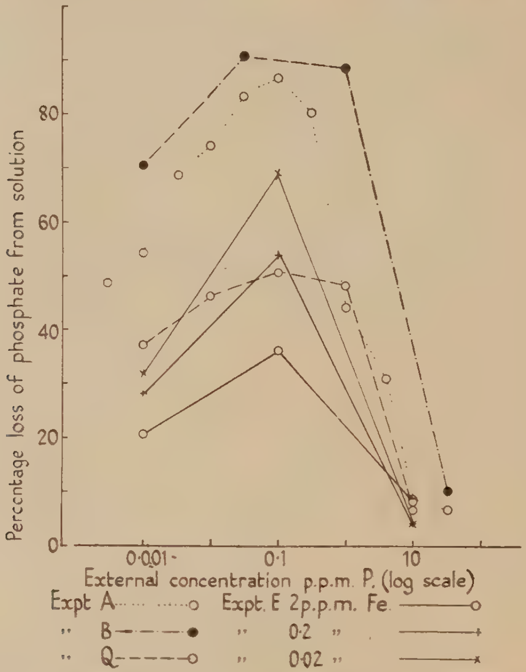

FIG. 5. Percentage of phosphate lost from solution in experiments of 24 hours duration.

TABLE II

Experiment R: *The effect of varying concentrations of phosphate on the absorption of phosphate by young barley plants. Duration of experiment*, 48 hours

	Initial concentration of external solution p.p.m. P			S.D.
	0·31	0·031	0·0031	(P = 0·05)
Relative absorption . . .	97·00	95·92	95·21	0·25
Phosphate remaining in solution after 48 hours expressed as a percentage of phosphate originally applied	2·76	3·69	4·23	0·62

formula appears adequate for demonstrating the general relationship between the external concentration and absorption.

In Fig. 4 results calculated by formula *iii* are given for other experiments in which plants were treated with labelled phosphate for 24 hours. In each experiment well-marked maxima are again shown at intermediate concentrations. Considerable quantitative differences, however, occurred. The three levels of iron employed in Expt. F markedly affected relative absorption. Between experiments there were even wider differences; this, it is considered, is attributable to the fact that seasonal changes in the environment of the greenhouse in which the work was carried out caused the size of the plants and their rates of growth to vary widely between experiments. For this reason a detailed comparison of the curves obtained in different experiments is inappropriate.

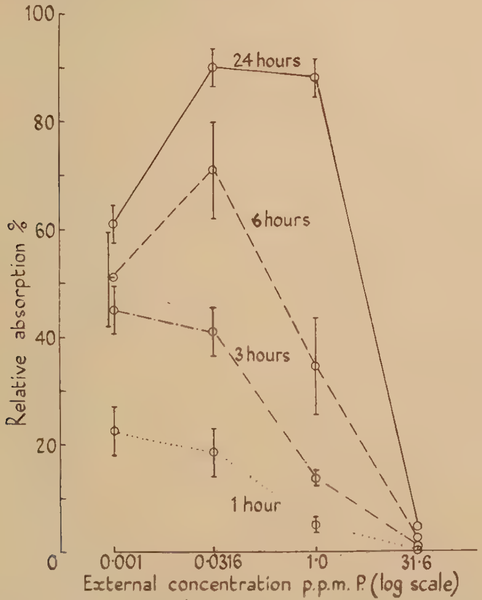

FIG. 6. Expt. B: Relative absorption over different periods: 5% fiducial limits are shown.

Confirmatory evidence that the proportion of the nutrient absorbed by the plants was greatest from solutions of intermediate concentration is provided by an examination of the extent to which the solutions were depleted (Fig. 5). It will be seen that in each case depletion was greatest from solutions of intermediate concentration.

In Expt. R the treatment period with labelled phosphate was extended to 48 hours. Although the external solution was exhausted to the extent of over 90 per cent. in all treatments, absorption was affected in the same manner as in the experiments already described; both relative absorption and the extent of depletion of the external solution decreased as the initial concentration was reduced from 0·31 to 0·0031 p.p.m. P (Table II).

Hitherto consideration has been confined to observations of absorption over periods of 24 hours or longer. In Expt. B, however, plants were sampled after treatment for 1, 3, and 6 hours as well as after 24 hours. The data for relative absorption on these occasions are compared in Fig. 6. On the first two sampling occasions no indication of a fall in relative absorption at th lower concentrations was shown. After 6 hours, however, the maximur value for relative absorption occurred in the 0·0316 p.p.m. P treatment an in the 24 hours treatment this effect was somewhat more marked; on bot occasions the values for the 0·001 p.p.m. P treatment were significantly belo those for the 0·0316 p.p.m. P treatment. It therefore appears that in the earl stages of absorption the absorption curve is not sigmoid in form, but that th characteristic develops with time.

The physical form of phosphate in dilute solutions

It is well known from radiochemical studies with very dilute solutions tha a significant fraction of the solute is frequently not in true solution; adsorption on to dust particles or the formation of radiocolloids (Schubert and Conr 1949) may be responsible for such effects. Phosphate solutions of the type used in the present investigation were centrifuged for 20 minutes at approximately 2,500 g. both before and after plants were grown in them. Negligibl quantities of phosphate were lost from solutions containing 0·1 p.p.m. P c higher concentrations of phosphate. A considerable fraction of the phosphat was, however, removed from solutions containing 0·001 p.p.m. P. For convenience, phosphate removed by centrifuging will be here called 'particulat phosphate' while phosphate not removed in this manner is regarded as bein in true solution. An examination of solutions in which no plants had bee grown showed that the formation of particulate phosphate was not dependen on the presence of plants in the solution, and that the amount of phosphat removed by centrifuging from solutions originally containing 0·001 p.p.m. rose from up to 10 per cent. shortly after the solutions were prepared to ove 40 per cent. 24 hours later. The addition of unlabelled phosphate or othe ions to dilute solutions of labelled phosphate led to a decrease in the activit of the particulate phosphate. The addition of the detergent 'teepol', or boilin for 5 minutes, brought about a similar effect. Dilution with distilled water increased the amount of phosphate in particulate form. These effects are illustrated in Table III. It is noteworthy that despite the fact that all treatment except dilution led to a reduction in the amount of particulate phosphate, th total phosphate content of the solution decreased simultaneously due to adsorption on to the walls of the vessels.

At an early stage in the work it was suspected that bacteria might b responsible for the removal of phosphate from true solution The foregoin results are, however, incompatible with that interpretation; they sugges rather that the adsorption of phosphate on to dust particles in the solutions i a more probable explanation of the formation of particulate phosphate. Sinc the release of phosphate from the particulate form occurred simultaneousl with losses by adsorption in the experiment described in Table III, phosphat must have been less strongly retained on the dust particles than by adsorptio on to glass.

TABLE III

he effects of various treatments on the particulate phosphate in a solution riginally containing 0·001 p.p.m. P *of which 41·9 per cent. was particulate at the beginning of the experiment*

Samples analysed 24 hours after treatment

Treatment	% change in amount of particulate phosphate	Particulate P / Total P in final solution %	Phosphate lost due to adsorption on to walls of vessels (% of initial value)
ontrol (no treatment) . .	+1·0	79·3	47·8
oiled 5 minutes . . .	−72·4	14·5	13·2
ddition of teepol (9% soln.) .	−66·1	23·4	16·0
,, 10^{-3}M KH_2PO_4 .	−63·4	25·8	16·6
,, 10^{-3}M KNO_3 .	−52·7	39·4	22·7
,, nutrient soln. .	−12·5	51·0	33·0
iluted 50% with distilled water	+41·4	89·4	34·2

The ability of plants to absorb particulate phosphate was examined by lacing them for 3 days in dilute solutions of labelled phosphate which conained high percentages of particulate phosphate. In Table IV the results are hown for two experiments in which the amount of phosphate in true solution as compared with the amount of phosphate absorbed by plants. In Expt. S e phosphate absorbed by the plant was 165 per cent. of the phosphate riginally present in true solution. Expt. T was similar to Expt. S except at two batches of plants were treated with the same solution in successive -day periods. In each period the decrease in the phosphate in true solution epresented less than half of the phosphate absorbed by the plants. Phosphate hich had been present in a particulate form had therefore been absorbed.

TABLE IV

he absorption of labelled phosphate by barley seedlings from solutions containing high percentages of particulate phosphate. Duration of experiments, 3 days

	Experiment S	Experiment T	
		1st period	2nd period
nitial solution: p.p.m. P . .	0·00075	0·00046	0·00015
% particulate	83·0	60·5	47·7
ctivity of P^{32} in true solution ($\mu C \times 10^{-3}$):			
Initial	77·2 ± 10·0	97·6 ±5·6	42·6 ±5·6
Final		42·6	29·9
Decrease		55·0	12·7
ctivity of P^{32} absorbed by plants .	127·1 ± 18·1	136·0 ± 29·6	27·5 ± 4·9

5% fiducial limits are shown.

When the solutions in series of vessels which had originally contained ·001 p.p.m. P were examined after absorption experiments, it was invariably found that the proportion of the phosphate which was in the particulate forn differed markedly between vessels. Thus it was possible to investigate th relationship between the amount of phosphate absorbed by plants and th percentage of the phosphate in the final solution which was in particulat form. Accordingly, the results were examined for 20 replicates from 2 experiments in which plants were supplied with 0·001 p.p.m. P for 24 hours. Th percentage of the phosphate in the final solution which was particulate varie from 70 to 86·6. No correlation was found between this quantity and absorption as measured by the ratio of phosphate in the plants to that in th final solution ($r = 0{\cdot}04$, value for 5 per cent. significance 0·51). This suggest that the physical form of phosphate in the external solution had no significan effect on the rate of absorption from dilute solutions.

Supporting evidence was provided by one of the experiments referred t in the preceding paragraph in which additional replicates had been supplie with 0·01 p.p.m. P. The ratio of phosphate absorbed to the total phosphat content of the final solution, and the ratio of particulate phosphate in tha solution to its total phosphate content for both treatments were:

	P absorbed × 100 / Total P in final solution	Particulate P × 100 / Total P in final solutior
0·01 p.p.m. P . . .	108·7	70·8
0·001 p.p.m. P . . .	48·7	83·0

An analysis of covariance on a logarithmic scale showed that the ratio c phosphate absorbed to that remaining in solution in the 0·001 p.p.m. P treatment exceeded the corresponding value for the 0·01 p.p.m. P treatment b 0·36±0·07, when adjustment had been made for differences in the percentag of particulate phosphate present in the two solutions.[1]

It may be concluded therefore that the fall in the relative absorption c plants when the external concentration of phosphate is reduced below 0· p.p.m. P cannot be attributed to the form of phosphate in the externa medium.

DISCUSSION

The novel feature of the present findings is that the relationship betwee the external concentration and the absorption of phosphate by intact plant is sigmoid when the concentrations employed range from 0·001 p.p.m. P t above 1 p.p.m. P and the duration of the observations is 12 hours or longe In consequence the values for relative absorption show a well-marked maximum when the external concentration is in the order of 0·1 p.p.m. P. Thi effect cannot be dismissed as resulting from the depletion of the culture solution or from adsorption losses on to the walls of the vessels employed, neithe can it be attributed to the physical form of phosphate in dilute solution In seeking an interpretation it is relevant to consider the results given in th preceding papers of this series (Russell and Martin, 1953; Russell, Martin, and Bishop, 1953). The percentage of the absorbed phosphate found in shoots (the 'transport index') decreased progressively as the external concentration was reduced below 0·1 p.p.m. P. It was considered that this effect was due to progressively larger fractions of the phosphate entering the plant being retained by metabolic processes in the root when the external concentration was reduced. The extent of such metabolic utilization was greatest in plants of low initial phosphate status. Furthermore, it was shown that when plants which had been previously treated with labelled phosphate were grown in phosphate-free solutions, the proportion of labelled phosphate which they ost to the outer medium was greatest in those which had previously been treated with the most dilute phosphate solutions. By contrast, plants which had been treated with 0·1 p.p.m. P or higher concentration of phosphate lost relatively small fractions of the previously absorbed phosphate. The marked oss of phosphate from plants treated with dilute solutions was regarded as ndicating that phosphate which had been retained in metabolic systems in he root was subsequently released and that a significant fraction diffused outwards. In Fig. 7 the relationship between relative absorption and both the transport index' and the percentage of labelled phosphate lost by the plants on transference to phosphate-free solutions is shown for the plants in Expt. A which received 0·1 p.p.m. P or lower concentrations. The levels of phosphate

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[1] The writers are indebted to Miss M. A. Creasy of the Lectureship in the Design an Analysis of Scientific Experiment, Oxford University, for the statistical analysis in this sectio of the paper.

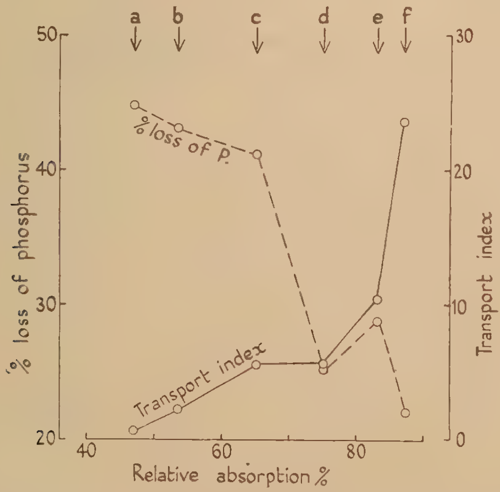

FIG. 7. *Experiment A*: Relationship of relative absorption to transport index and percentage of phosphate lost on transference to phosphate-free solutions. The arrows indicate the concentrations of labelled phosphate applied in the initial period of the experiment. *a*, 0·000316 p.p.m. P; *b*, 0·00100 p.p.m. P; *c*, 0·00316 p.p.m. P; *d*, 0·0100 p.p.m. P; *e*, 0·0316 p.p.m. P; *f*, 0·100 p.p.m. P.

supplied are indicated in the figure. These results suggest that the reduction in relative absorption when the external concentration is reduced below 0·1 p.p.m. P may result from a greater proportionate loss of recently absorbed phosphate in consequence of its release from metabolic processes in the roots. This interpretation, which does not imply any reduction in the relative rate of primary absorption, is supported by the fact that in periods of 1 or 3 hours relative absorption was maximal in plants supplied with the most dilute solutions (Fig. 6).

The factors which affect the form of the absorption curve may now be considered. Numerous workers have examined this relationship in comparatively concentrated solutions, and the curve has been shown to resemble an adsorption isotherm (e.g. Helder, 1952). The concentration at which the curve becomes asymptotic at its upper end is, however, affected by many factors. van den Honert (1933) showed that, while at pH 4·5 the maximum absorption of sugar-cane occurred when the external concentration was 1 milli-equivalent P_2O_5/litre (i.e. 31 p.p.m. P), at pH 7·5 absorption increased until twice that concentration was reached. Alberda (1948) showed that 'high salt' plants attained their maximum rate of uptake at a lower concentration than 'low salt' plants. Helder (1951, 1952) stressed the importance of growth in determining the absorption of phosphate and showed that the addition of glucose to 'low salt' plants may increase the concentration at which the maximum absorption is attained. The shape of the lower part of the absorption curve is likewise dependent on many factors. If the interpretation of the physiological causes of the decrease in relative absorption here advanced is correct, all factors which increase the 'transport index' will tend to reduce the sigmoid nature of the curve. It has been shown (Russell, Martin, and Bishop, 1953) that the transport index of plants supplied with low concentrations of phosphate can be increased by raising their initial phosphate status. The same effect was induced by intermediate concentrations of respiratory inhibitors; the extent of absorption was in some instances thereby increased.

The relationship between the absorption of phosphate by intact plants and the external supply can therefore not be susceptible to any simple mathematical analysis if a wide range of concentration is used. During the course of an experiment high concentrations, i.e. those in excess of 1 p.p.m. P, will change the phosphate status of plants significantly and to different extents at each concentration; this will affect the form of the curve. In the lower range of concentration, changes both in the initial phosphate status of the plants and in the duration of experiments can profoundly alter the relationship.

A point of special interest is whether the absorption curve passes through the origin or whether there is a lower concentration threshold for the absorption of phosphate as Olsen (1950) has suggested. His statement that plants cannot absorb phosphate from solutions containing less than 0·0003 m.e./l. (i.e. 0·0093 p.p.m. P) is clearly not supported by the present results; considerable absorption has been shown from solutions initially containing 0·000316 p.p.m. P, and it seems possible that Olsen's conclusions were due to the relative insensitivity of the chemical procedures for the estimation of phosphate which were available to him. Whether or not the absorption curve does pass through the origin must, however, remain in doubt since extrapolation may be misleading on account of the form of the curve.

In conclusion, reference may be made to the possible implications of these results from the viewpoint of the phosphate nutrition of plants when their growth is limited by the external supply of phosphate. Under such circumstances the placement of fertilizers close to the seed (Lewis and Strickland, 1944) or the treatment of seed with phosphate prior to planting (Roberts, 1948) may cause marked improvement in growth and phosphate content, though the latter treatment has not always been successful (Rhind *et al.*, 1949). One obvious reason for the beneficial effects of these procedures is that the increased phosphate content of the plants induces more rapid root growth and thereby enables the plant to absorb from a wider soil zone; the importance of this is obvious in view of the immobility of phosphate in soil. It is probable also that such treatments lessen the extent to which phosphate is converted into sparingly available forms in the soil. The present findings suggest, however, that the increased growth resultant from fertilizer placement or seed treatment may be in part due to the increased phosphate status of the plant causing a larger proportion of the absorbed phosphate to be translocated to the shoots, and to the rate of absorption per unit of root thereby being increased; both these effects would favour the expansion of the photosynthetic area.

ACKNOWLEDGEMENTS

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A New Theory of the Mechanism of Stomatal Movement

W. T. WILLIAMS

Department of Botany, The University, Southampton

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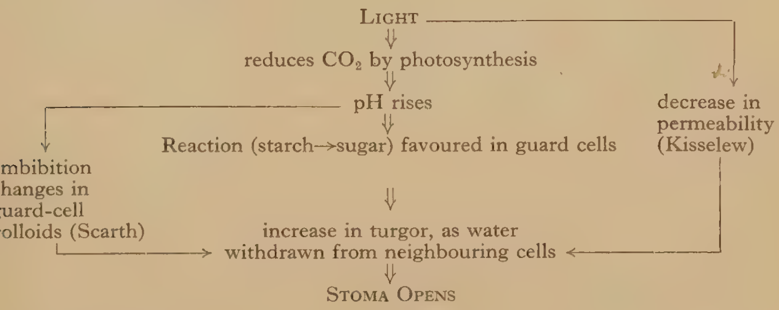
SUMMARY

After a brief examination of the classical theories of stomatal movement and their drawbacks, a new theory is propounded. It postulates (*a*) that the important movement is that of *closure*; (*b*) that this is an 'active' process; and (*c*) that the ubiquitous carbohydrate changes are secondary, 'stabilizing' changes, probably not directly linked to the main process. The evidence for the theory is stated and discussed, and some implications examined.

INTRODUCTION

CONSIDERABLE amount of work on the physiology of stomata has been ublished since the last classic review by Scarth (1932), much of it by Dr.). V. S. Heath and the present writer. Yet, despite the not inconsiderable ody of knowledge that this work has produced, its effect on stomatal theory as been predominantly negative: it has taught us more about the mechanisms y which stomata do *not* work than about any by which they do. I feel that here is once again room for a new positive theory which, however implausible r insecurely based, may yet provide an impetus for new lines of approach. The purpose of this paper, then, is to provide such a theory, together with uch scanty evidence as can justify it; but first we must examine briefly the resent state of stomatal theory.

The classical theory. This is in no sense intended to be a comprehensive eview, and simplification is therefore inevitable; with this proviso, we may ay that the most commonly quoted theory of stomatal movement is in essenials that of the centre path (reading downwards from 'light' to 'stoma opens') n the following scheme:


LIGHT
reduces CO_2 by photosynthesis
pH rises
Reaction (starch→sugar) favoured in guard cells
increase in turgor, as water withdrawn from neighbouring cells
STOMA OPENS
mbibition hanges in uard-cell olloids (Scarth)
decrease in permeability (Kisselew)

This scheme has no prima facie improbabilities and is internally consistent;

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it is, roughly, the theory accepted by Sayre (1926) and by Small and hi colleagues (1939, 1942, 1950). It has, though, always had its critics. The association of carbohydrate changes with movement is so well established tha all writers are agreed that such changes must play *some* role; but difficultie have arisen in accepting them as the primary cause of movement. The objection has sometimes been that starch changes are too slow to account for th rapidity of movement; but Small *et al.* (1942) (for hydrolysis) and William and Barrett (1954) (for synthesis) have shown that this objection is untenable The more usual, and I believe unanswerable, objection is that carbohydrat and aperture changes, though roughly correlated, are not exactly so, and th theory demands that they must be (Kisselew, 1925; Scarth, 1932; Heath 1949, 1952; Williams *et al.*, 1952, 1954). Kisselew propounded in consequenc his theory of permeability changes; but his results are far from convincing based as they are mainly on visual estimates of the degree of entry of indigo-carmine, and attempts by later investigators to repeat his observations wer inconclusive. Scarth similarly propounded his theory of pH-activated swellin of guard-cell colloids; the only direct investigation along these lines of whic I am aware is that by Alvim (1949), who was unable to confirm Scarth's suggestion. Nadel (1935), who is sometimes credited with a related theory to th effect that movements are brought about by changes in the degree of swellin of the walls, was concerned primarily with post-mortem movements in variou liquids; she acknowledged the importance of turgor changes in the livin stoma, suggesting merely that changes in the walls might somewhat modif the process. Any lingering doubts as to the primacy of turgor changes wer in any case dispelled by the experiments of Heath (1938).

Nor are the pH changes themselves beyond dispute. The original observations of Sayre were carried out with indicators taken up into the guard-cel vacuole; but, since a fairly high concentration within the guard-cell would b needed for a colour-change to be discernible, and since indicators are necessarily strongly dissociable substances, the conditions within the guard-cel must have been highly abnormal. Moreover, the careful researches of Freudenberger (1940), and especially of Heath *et al.* (1950, 1950), have shown tha the greater part of the range of stomatal movement occurs within the rang 0·01 per cent. to 0·03 per cent. of CO_2 by volume in the intercellular spac (I neglect at this stage possible *direct* effects of light); and so small a chang in CO_2-content can hardly be conceived as directly bringing about large p changes. Indeed, as I have previously pointed out (Williams, 1949), the effec is strongly reminiscent of some of the phenomena of animal respiration, i which CO_2 appears to act *per se* as a metabolically active substance. I have no forgotten the many experiments carried out on epidermal strips immersed i buffer solutions; but I give below my reasons for believing that, valuabl though these are in themselves, they do not at present necessarily assist in th elucidation of the phenomena of the intact leaf.

The classical theory, then, is not entirely satisfactory; while modern wor has not refuted it, it has been unable to support it.

A NEW THEORY

The theory may be summarized as follows: (i) The important movement is the *closing* movement; opening being a return, probably osmotic, to a resting state. (ii) This closing is an 'active', non-osmotic, energy-requiring transfer of water from guard-cell to neighbouring cells, possibly mediated by contractile structures of some type. (iii) Carbohydrate changes are secondary, 'stabilizing' changes, not directly linked to the primary process.

We will examine these three aspects in turn.

The closing movement. Historically, the concentration of attention on the opening movement is probably due to the fact that light opens stomata; light, being an energy supply, is a *positive* factor, and, if it is to have an effect, that effect must logically be activated by its presence and not by its absence. However, Heath has shown conclusively that light acts by virtue of the *absence* of CO_2 that it engenders; and darkness acts by virtue of its presence. I am aware that Heath and Russell (1954) have shown that there is a direct effect of light on the guard-cells; but, since the species for which it was shown possesses chloroplasts in the guard-cells, it is possible that light in this case acts via intracellular CO_2 change, within the guard-cell. Heath and Russell give reasons for believing that this is not the case; but, until the phenomenon has been further elucidated, we may at this stage (*vide* p. 347 below) accept the principle of Occam and postulate that *all* light-induced movements are in fact induced by a reduction in CO_2, and the situation is thus completely reversed. CO_2 is metabolically a very active substance; its presence may be expected to have a positive effect and the important movement is thus likely to be of that closure. But is there any evidence for this?

There is, I think, a little; and I would draw attention to the following phenomena:

(*a*) Opening is a fairly rapid process—the major part of the range in *Pelargonium* is completed in about 30 minutes; closure normally takes a comparable time. But, as I have elsewhere shown (Williams, 1950), it is possible by manipulation of the external conditions to produce a remarkable acceleration of the closing reaction—after prolonged exposure to dry air, the closing time can be reduced, once closure has begun, to something of the order of 2 minutes. In fact, with an extreme case of the phenomenon, the closing reaction can only be followed by taking 10-second readings; but the opening reaction is unchanged. The closing reaction evidently shows a profound sensitivity to abnormal external conditions.

(*b*) In the course of mechanical shock experiments (Williams, 1951) I observed (but did not publish the observations) that the closing curves which followed severe mechanical stimulation were often irregular or otherwise abnormal in form; this was particularly true of responses followed 2 or 3 days after the shock, as if a delicate mechanism had been damaged and was deteriorating. However, these irregularities were virtually confined to the closing movement; the opening curve, if it could be obtained at all, was substantially normal.

(*c*) One of the rare investigations into stomatal dynamics was carried out by F. W. Cope in Professor F. G. Gregory's laboratories at Imperial College; the results have not yet been published, and I am much indebted to Professor Gregory for permission to quote from them. Briefly, Cope was investigating the reversal of stomatal movement by the sudden darkening of opening stomata or the sudden illumination of closing stomata. If stomata in the course of opening were darkened, the effect, at any stage of the process, was much the same; a slight 'overshoot', then a reversal of direction. On reillumination there was again a slight 'overshoot', after which opening proceeded as before. However, when a *fully* open stoma, which had begun to close in darkness, was reilluminated, the situation was very different: the 'overswing' carried the curve well up towards closure before it fell again. The closing reaction, once properly embarked upon from the fully open position, cannot be rapidly reversed by reversing the external conditions.

The primary mechanism. It is accepted that stomatal movements are primarily the result of turgor-changes; but the more spectacular turgor movements (the shock-reaction of the sensitive species of *Mimosa*, the autonomic movements of *Desmodium gyrans* and *Averrhoa*, the nyctinastic movements so well shown by members of the Leguminosae and Oxalidaceae) share a curious distinction: until very recently, in no single case was the mechanism understood. The great difficulty has always been the speed with which water is lost from the pulvinar cells, a speed hardly consistent with the known high resistance of protoplasmic membranes to the passage of water (*vide*, e.g., Mer, 1940; van den Honert, 1948, and references therein cited). It appears to be impossible to invoke permeability-changes; for not only does the liquid lost appear to contain, at any rate, no electrolytes (Blackman and Paine, 1918), but it is difficult to imagine how the process could be quickly repeated if osmotically active substances were lost at every pulvinar discharge -the osmotic gradient would surely soon become reversed and the cells incapable of reassuming their original relationship.

The first convincing explanation of movement in *Mimosa* has recently been given by Weintraub (1951), whose claim that water is extruded via contractile vacuoles is supported by much detailed observation. There should be nothing surprising in this suggestion; this is the method by which water is moved by many protozoa and unicellular algae; being energy-assisted, it allows for the movement of water as distinct from solution, independent of osmotic gradients; and the speed may be considerable, since the normal membrane arrangements are, at least in part, by-passed. The concept of vacuoles which appear only in response to stimulation is novel, but an organization of numerous minute vacuoles rather than a few conspicuous ones is already familiar to us from, for example, *Sphaerella lacustris*. Note particularly that the active movement is that associated with *loss* of turgor.

We may now examine some remarkable parallels between the movements of 'sensitive' plants and of stomata:

(*a*) Stomata are very insensitive to mechanical shock (Williams, 1949, 1951), but if they react at all they close. Admittedly the phenomena concerned can all be plausibly accommodated within a CO_2-change hypothesis; but still, it is *closure* which corresponds with the shock-movement of *Mimosa*.

(*b*) In the darkness the pulvinar cells of *Mimosa*—and, for that matter, all similar nyctinastic plants—lose their turgor, the process being presumably similar to, though gentler than, the shock-reaction. In the darkness, stomata close.

(*c*) Certain turgor movements in sensitive plants—the so-called 'movements of variation'—show strong autonomous rhythms; the movements of *Desmodium*, *Eleiotis*, *Averrhoa* continue rhythmically in continuous light if the temperature is appropriate, and may continue for a time in darkness (*vide*, e.g., the account in Stiles, 1936). The movements in *Mimosa* are normally tied to diurnal changes in light intensity, though they will continue for a time in continuous darkness. The movements of stomata are similarly normally associated with diurnal changes (for the many variants possible within this general pattern, *vide* Darwin, 1898, and Loftfield, 1921); but there is evidence for *Pelargonium* that diurnal movements of small amplitude occur in continuous light, upon which may be superimposed autonomic pulsations of shorter period (Gregory and Pearse, 1937; Williams, 1952). Precisely similar results, but based on far more extensive evidence, were obtained by Solovsky-Irtel (1934) for *Impatiens sultani* and *Sedum pachyphyllum*.

(*d*) The shock-induced loss of pulvinar turgor in *Mimosa* is so rapid that, as I have pointed out above, it is scarcely conceivable that the normal considerations of protoplasmic resistance to water-movement can apply; the re-establishment of turgor—significantly always referred to as 'recovery'—may be rapid, but never attains the catastrophic proportions which have gained for seismonasty its name. Under the special conditions of prolonged exposure to a dry external atmosphere, the closing movement of stomata can similarly show this extraordinary speed of loss of turgor; but the consequent opening movement—should it, too, be called 'recovery'?—is unaffected.

(*e*) A more subtle problem arises in connexion with both nyctinastic plants and stomata. If dark-induced loss of turgor is an energy-assisted process, how, during the ensuing hours of darkness, is it maintained? Must water be continuously pumped out to maintain the flaccid condition? It would appear so: for the theory postulates that the turgid condition is, osmotically, the position of rest, to which the cell must return if contractile forces are withdrawn—as occurs in the light, or even, in response to an autonomic stimulus, in the unchanging darkness. (This effect—that the closing reaction can be suppressed in the absence of external change—may perhaps be related to the direct effect of light on guard-cells reported by Heath and Russell, 1954). However, in the case of stomata the situation is complicated by the apparently ubiquitous carbohydrate changes; and to these we must now turn our attention.

The carbohydrate changes. The hypothesis that carbohydrate changes represent a secondary, and perhaps independent, mechanism seems first to have been advanced by Kisselew (1925). I have elsewhere resuscitated it (Williams,

1952) to account for the fact that the patterns of response of starch and aperture to external conditions appear to be quite different (*vide* references *supra*), though the classical imperfect negative correlation may arise as a combination of these two patterns (Williams and Barrett, 1954). By exposing a leaf to a dry external atmosphere the carbohydrate change can be suppressed, thereby (according to the views here advanced) leaving the 'active' mechanism in full control; the resulting extraordinary acceleration of the closing movement has already been mentioned. Starch itself shows a diurnal rhythm (Heath, 1949; Williams, 1952) and it is noteworthy that *Pelargonium* stomata react more rapidly to darkness in the late afternoon (when starch is high) than in the morning (when it is low). Even after prolonged darkening, stomata open very rapidly on illumination: different workers have reported that movement is discernible within times such as 5 seconds or 1 minute of illumination. The onset of closure almost always shows a lag after darkening if the stomata are fully open; it is as if the wall is most stable in the open position, so that a purely mechanical resistance has to be overcome. However, if stomata have been allowed to open wide in an unswept porometer-cup, so that the starch in the guard-cells is greatly reduced as a result of the combined effects of high humidity and low CO_2 (Williams and Barrett, 1954), this lag is often greatly increased, the stoma appearing to be temporarily 'stuck' in the open position. If starch decrease is in fact associated with an increase of osmotic substances, could it not be that the osmotic gradient is too high even for the 'active' system to overcome, so that movement cannot begin until starch is resynthesized in response to the rising CO_2 concentration in the darkness? I find that these observations are at least consistent with Kisselew's view that the carbohydrate changes represent an 'amplifying and stabilizing' process which *usually* follows the light-induced aperture changes; and which, if my views are correct, serves to prevent the stomata from behaving in the disconcerting—and, for stomata, biologically undesirable—manner of *Mimosa*.

It is impossible at this point to evade the problem of the carbohydrate and aperture changes which occur when epidermal strips are immersed in solutions. I have no wish to belittle the mass of data on this subject collected by Arends (1926), Small *et al.* (1939, 1942), Saïd and Tolba (1948), and Tolba (1952); I do, however, doubt whether the process being studied is the same as that which occurs in the intact leaf. The erratic nature of the results, very clear from the graphs of the later workers; the dependence on the kind, rather than the pH, of the buffer solution used; the dramatic light-controlled 'sodium acetate effect' of Williams and Shipton (1950)—all these are difficult to relate to normal movements. It might be that we have here a situation in which the 'active' movement is suppressed, so that the normally subsidiary osmotic changes have assumed primary responsibility for movement, the effects of the solutions being on the enzymes concerned with carbohydrate change; it is difficult to reconcile this theory with the observations by Arends (1926) that in dilute alkalis starch decreases markedly and the stomata close, but some recent experiments by Amer (in preparation) seem likely to resolve the apparent contradiction. The behaviour of stomata in stripped epidermis is associated with two special problems, the first of which is that in an intact leaf stomatal movement can take place only within a narrow range of epidermal turgor. Von Mohl (1856) noted that, if whole leaves were immersed in water, the stomata closed in all cases examined, except members of the Orchidaceae, whose stomata opened; similar observations have been made by Darwin (1898), Stålfelt (1929), and, on isolated stomata, by Mouravieff (1951). The phenomenon, so far as is known, is purely mechanical, and rather difficult to reconcile with the familiar observation that in a saturated atmosphere, as distinct from liquid water, stomata are difficult to close. The second problem is that stripping an epidermis tears open, in most cases, a high proportion of the epidermal cells, so that normal turgor relations are again upset —quite apart from the possibility that, if there *is* an active or contractile system, the recipient cell might itself participate in it. In the circumstances, it is not surprising that the behaviour of stomata in epidermal strips is aberrant and its interpretation difficult; it is, I believe, a separate problem which may well prove more intractable even than that associated with normal movement.

FURTHER IMPLICATIONS OF THE THEORY

The most useful property of a new theory is that it suggests new experiments. It is virtually impossible to undertake any scientific research without some underlying concept or hypothesis, conscious or unconscious, which confers relevance and meaning on the experiments (for a discussion of this principle, *vide*, e.g., Baker, 1934). The advantage of the theory under discussion is that, being fundamentally unlike previous theories of stomatal mechanism, it suggests experiments markedly different from those normally undertaken.

It has, for example, been pointed out to me by Professor Meirion Thomas that, if stomatal closure is in fact mediated by an active mechanism (such as contractile vacuoles), it is likely to require oxygen. This aspect has been little investigated, since earlier theories do not lead us to expect such a phenomenon. There is, though, the observation by Gregory and Armstrong (1936) that the stomata of *Pelargonium* could not be closed in an atmosphere of hydrogen, although the effect was not shown by *Fuchsia*. This might, of course, be merely a 'lack of CO_2' effect; but might the stomata of *Pelargonium* have a higher O_2 requirement for closure than those of *Fuchsia*? More convincing support for the theory comes from the observations of Freudenberger (1940), who specifically investigated stomatal behaviour in the absence of oxygen. She found, first, that in darkness the effect of supplying *Canna* or *Impatiens* leaves with nitrogen completely freed from oxygen was that the stomata first closed, then slowly opened again. She points out that the closure was doubtless a normal reaction, carried out by virtue of O_2 still remaining in the intercellular atmosphere. The subsequent opening she was unable to explain; but on my theory it is to be expected if the system (as is likely) is O_2-sensitive. Secondly, she found that the effect of adding CO_2 to the N_2 was to cause a marked further *opening*—a complete reversal of the normal CC effect. This, too, is to be expected on my theory in view of the CO_2-concentr tions employed. For *Sanchezia* she used equal parts of CO_2 and N_2. No CO_2 at this concentration is not merely suffocating, but toxic; for m it becomes an irrespirable gas well below this level. Such a concentrati would be expected to suppress immediately any remaining activity still p sible to the system, and the stomata would open. The same effect was shov by *Canna* at the lower, but still high, concentration of 10 per cent.; it wou be most interesting to discover the concentration at which it disappears.

Then there are the respiratory inhibitors: azide and the rest. If the co clusions of La Rue (1930), Wylie (1943), and Armacost (1944) are correct, a water passing up a petiole permeates the epidermis of a leaf before it ente the mesophyll, it might be possible to introduce inhibitors via the petiole a affect the stomata before the leaf as a whole is damaged. The zoological liter ture on the physiology of contractile vacuoles would surely suggest new lin of investigation; so might that dealing with biological rhythms (*vide*, e.g., t brief review by Kalmus (1953) of a recent conference on this subject). The should, too, be a temperature effect; since practically all aspects of metabolis are affected by temperature, the relationship is unlikely to be simple; but would be likely that, under some conditions at least and over a restrict range, a rise in temperature should accelerate closure. Most workers (*vi* e.g., Darwin, 1898, and Wilson, 1948) seem to have found that a high temper ture favours opening; but the expected result has been reported by Hea (1952) for *Allium*. It must, however, always be remembered that from t point of view of this theory temperature is likely to affect, not the equilibriu values of aperture (which may be temperature-sensitive for other reason but the *rate* at which such apertures are attained.

This raises perhaps the most important point of all. This theory deman an investigation into the *dynamics* of stomatal movement—a study of rat rather than of final positions; for it postulates that the opening and closi reactions are fundamentally different in nature, and is not concerned with t equilibrium values attained. My own early investigations (Williams, 194 though not justifying wider publication, at least suggested that there was so real difference between the two movements, since they could not be fitted wi the same mathematical curve; these, the as yet unpublished experiments Cope previously referred to, and the brief investigation by Gregory ar Pearse (1937) into behaviour in intermittent light, are among the few rece investigations of this type. There is a good reason for the dearth of su investigations. They demand a porometer, and the critical examination of t resistance porometer (Gregory and Pearse, 1934) by Heath (1941), Spanner ar Heath (1951), and Penman (1942) has shown how complex and subtle a quanti is a porometer reading; and dynamic observations are consequently so difficu to interpret that they are seldom undertaken—or, if undertaken, seldom pul lished. Though this theory may have no other virtue, if it directs attention on more to the study of stomatal dynamics, it will not have failed completely.

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Detection of Glycollic Acid in Etiolated Barley Shoots

DAPHNE C. ELLIOTT
Department of Botany, University of Oxford

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SUMMARY

A modified method for carboxylic acid analysis has been developed in order to study the metabolism of glycollic acid in barley sap.

Glycollic, malic, citric, malonic, succinic, and fumaric acids have been detected in alcoholic extracts from etiolated barley shoots, and the amounts present roughly estimated.

INTRODUCTION

THE formation of glycollic acid in barley has been associated with photosynthesis (Burris *et al.*, 1949; Benson and Calvin, 1950) and has not been reported in etiolated shoots. Glycollic oxidase also has been found only in green tissues (Tolbert and Burris, 1950), and this is attributed to adaptive enzyme formation due to glycollic acid production during photosynthesis (Tolbert *et al.*, 1951).

The present work demonstrates chromatographically the presence of glycollic acid in shoots of barley germinated in the absence of light. For purposes of comparison, some of the other acids present have been included in this study.

The method of Bryant and Overell (1951, 1953) for the chromatographic analysis of carboxylic acids in plant tissues has been modified to allow the detection of acids which are present in amounts too small for detection by the original method. The preliminary use of a cation exchange resin, Zeo-Karb 215, has been introduced to adsorb amino-acids which otherwise become held on the anion exchange column of Amberlite IRA 400. In addition, the dried ammonium carbonate eluate has been extracted with ether because of a certain amount of unvolatilized residue which remains on drying, and which so interferes with subsequent chromatography that it is impossible to detect carboxylic acids which are present only in traces (like fumaric, α-ketoglutaric, and aconitic acids). For the detection of malic and citric acids this ether extraction step is not necessary.

EXPERIMENTAL

Preparation of columns. Zeo-Karb 215 was treated as described by Partridge and Westall (1949). Material of particle size 40–100 mesh/inch was used for making up the column (height, 10 cm.; internal diameter of tube, 1·0 cm.),

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which, after final activation with 2N HCl, was washed with glass-distilled water until the effluent was free from Cl^-.

Amberlite IRA 400 was dried at 40° C. for 24 hours, ground in an electric mill, and sieved. Material of particle size 60–100 mesh/inch was treated with 0·3 N HCl until there was no further evolution of gas. The supernatant was then decanted and the resin soaked for 1 hour in 4 vols. 0·3 N HCl. The column, containing 8 g. air-dry resin (height, 12 cm.; internal diameter of tube, 1·0 cm.), was made up and washed until the effluent gave only a slight reaction for Cl^-. The resin was activated by allowing 50 ml. M Na_2CO_3 to percolate slowly through (0·5 ml./min.) and then washed with glass-distilled water until the pH of the effluent had dropped below 7·0.

Preparation of extract. 20 g. etiolated shoots of 10-day-old barley seedlings (variety 'Spratt Archer') were homogenized with 80 ml. 80 per cent. ethanol in a Waring Blendor. The homogenate was squeezed through muslin and the extract filtered. The filtrate (83 ml., pH 5·2) was allowed to flow slowly through the two ion exchange resin columns, Zeo-Karb 215, followed by Amberlite IRA 400, at a rate of 0·5–0·8 ml./min.

Elution from Amberlite IRA 400. After washing through the columns with 25 ml. 50 per cent. ethanol followed by 25 ml. H_2O, the Zeo-Karb 215 column was discarded, and the acids eluted from the column of Amberlite IRA 400 by allowing 300 ml. M $(NH_4)_2CO_3$ to percolate through (0·5 ml./min.). Once the flow rate is steady, this elution can generally be allowed to proceed overnight. The eluate was evaporated to dryness on a water-bath. During the later stages of the evaporation, the solution tends to become acid, and there is some loss of glycollic acid. This can be prevented by the addition of KOH to keep the pH at 7·0.

Ether extraction of eluate residue. The residue was transferred as quantitatively as possible to a Soxhlet thimble. The ether extraction must be carried out at a pH of < 3, and to achieve this the evaporating dish was washed out with two lots of 0·5 ml. 2 N HCl, the acid being absorbed on small pieces of cotton-wool before being added to the residue in the thimble. The evaporating dish was then washed out with five lots of 25 ml. ether, the ether being added to the Soxhlet apparatus. Extraction was carried out for 22 hours. The ether was then evaporated from the flask, the residue taken up in water, neutralized by the addition of ammonia, and water added to bring the final volume to 5 ml. One-fifth of this solution (equivalent to 4 g. fresh weight shoots), run on a two-dimensional chromatogram, gives the picture shown in Fig. 1.

Paper chromatography. The solvents used were those of Brown (1951) and Lugg and Overell (1948), with slight modifications in composition. Chromatograms were first run in ethanol 15N NH_3 H_2O (80/5/15) overnight. This solvent travels approximately 50 cm. in 18 hours at 22° C., using Whatman No. 1 paper. The papers were dried for 30 minutes in a current of warm air, and then run in the second solvent, mesityl oxide 90 per cent. formic acid H_2O (200/41/200) for 4 to 5 hours. This solvent travels about 30 cm. in 4 hours at 22° C. on Whatman No. 1 paper. Papers were then dried in a current of warm air for 3 hours before spraying with bromophenol blue (0·04 per cent. in *n*-butanol, adjusted to a reddish-purple colour by the addition of NaOH).

A semi-quantitative estimation of the amount of carboxylic acid present was obtained by comparing the area of each spot with a reference curve obtained from chromatograms, run simultaneously, of known concentrations of the acids.

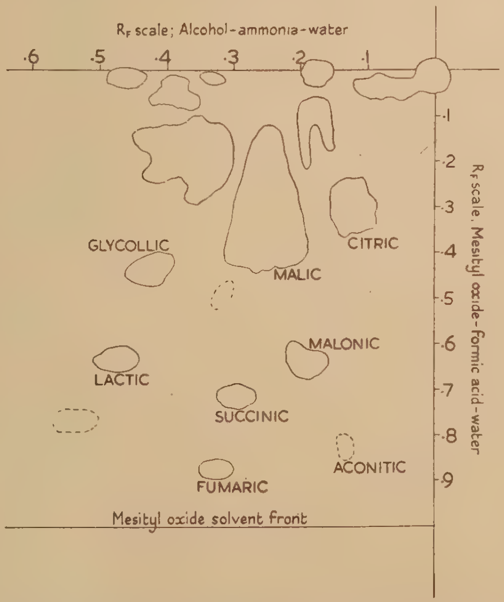
R_F scale; Alcohol-ammonia-water
·6
·5
·4
·3
·2
·1
·1
·2
·3
·4
·5
·6
·7
·8
·9
R_F scale. Mesityl oxide-formic acid-water
GLYCOLLIC
CITRIC
MALIC
MALONIC
LACTIC
SUCCINIC
ACONITIC
FUMARIC
Mesityl oxide solvent front

FIG. 1. Tracing of a chromatogram showing the carboxylic acids present in an 80% ethanol extract of etiolated barley shoots (equivalent to 4 g. fresh weight shoots).

The results given in Table I are the amounts of the carboxylic acids present in barley shoots as estimated by this method. Because of the loss of lactic acid from chromatograms during drying, no estimate can be given of the concentration of this acid.

TABLE I

Carboxylic acids in etiolated barley shoots

Acid	µg./g. fresh weight
Malic	370
Citric	35
Glycollic	15
Malonic	5
Succinic	5
Fumaric	3
α-ketoglutaric	Trace
Aconitic	Trace

Limits of detection. 10 μg. acid can be detected on these chromatograms, and a solution equivalent to 10 g. fresh weight leaves can be applied to one paper without overloading, so that concentrations as low as 1 μg. g. fresh weight can be detected by this method.

Percentage recovery. When a known mixture of carboxylic acids, in concentrations approximating to those present in 20 g. fresh weight barley shoots, is put through the procedure described above for barley extract, there is, within the limits of error of estimation on the chromatograms, a 100 per cent. recovery for all acids except citric and succinic (80–90 per cent.) and glycollic (70–80 per cent.) acids. No acids could be detected in the percolate from the Amberlite IRA 400 column, nor in the eluate when a second lot of 300 ml. $(NH_4)_2CO_3$ was run through the column. A re-extraction of the material in the Soxhlet for a further 22 hours gave rise only to a trace of malic acid (about 20 μg.) in the ether extract. The quantities of acids used in this recovery test were 7,000 μg. malic acid, 3,000 μg. each of citric and glycollic acids, 600 μg. malonic acid, 400 μg. succinic acid, and 300 μg. fumaric acid, but mixtures containing up to 20,000 μg. malic acid, plus 4,000 μg. of each of the other five acids, have been used on columns described above with similar recoveries. The column of Amberlite IRA 400 used in this work probably has a capacity for organic acids much greater than the total amount applied in the above recovery test (Bryant and Overell, 1951, 1953). An excess of resin was used to eliminate the danger of overloading the column with inorganic and other anions present in the barley extract.

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Experiments on the Growth and Geotropism of Roots

A. F. YOUNIS
Department of Botany, Faculty of Science, Alexandra University, Egypt.

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PART I. TECHNIQUE FOR ACHIEVING REGULAR GROWTH, AND A STUDY OF THE EFFECTS OF DECAPITATION AND REHEADING ON THE GROWTH OF *VICIA FABA* ROOTS[1]

SUMMARY

A technique is described whereby regular growth of roots is obtainable with good aeration.

Decapitation at 0·5, 1, or 2 mm. behind the root-apex does not accelerate the growth of the stump. An obvious retardation of growth is recorded when 2 mm. of the root-tip is removed, a less conspicuous retardation, or no effect at all, is recorded when shorter parts are removed. The amount of retardation is affected by the extent to which the apical meristem is removed.

The growth of stumps reheaded with their own tips is similar to that of unreheaded stumps.

INTRODUCTION

DECAPITATION has been frequently used as a means of detecting the role of the tip in the growth activity of the organ; and the value of this technique in verifying the claims of some theories such as the growth-hormone theory is widely acknowledged. With roots, the effects of decapitation on growth have been studied by various investigators, and the marked disagreement of results and claims prompted the present study.

Cholodny (1926), in his attempt to apply the growth-hormone theory to the case of roots, recalled earlier investigations of Wiesner (1881) and Rothert (1893) which gave support to the theory. His own experiments with *Lupinus angustifolius* showed that the decapitated roots grew more quickly than intact roots and that reheading caused a relatively small (about 5 per cent.) slackening of growth. Bünning (1928), on the other hand, found that in a number of plants the roots showed an initial retardation after decapitation. He also found that, if the tip *or any other part* of the root was placed on the stump, the reheaded root grew more slowly than the unreheaded stump. Bünning's conclusion from his experiments was, accordingly, that the root-tip formed no growth substance. These findings were contradicted by Cholodny (1929).

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[1] The experiments described in Part I were carried out in the Department of Botany, King's College, London, during 1947–50.

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The experiments of Keeble, Nelson, and Snow (1931), though often quoted as being in favour of the theory, led to no definite conclusion, a fact stressed by the authors' statement '. . . the results did not help toward determining the effect of the tip on growth, on account of the effect of wound-shock which seemed quite to obscure any effect due to loss of the tip.'

Gorter (1932), quoted by Cholodny (1933), found that decapitation did not affect the rate of growth of roots of *Pisum*.

In spite of this uncertainty about the effect of decapitation on growth, Went and Thimann (1937, p. 142) stated that '. . . if the experiments are carried out carefully and not too much of the tip is removed, all the roots studied—with the exception of those of *Pisum*—show a definite acceleration after decapitation'.

MATERIAL AND TECHNIQUE

Vicia faba (variety 'Windsor') was used in the present investigation. The experiments were performed in a dark room at constant temperature 25° C. For close-up observations, light from a hand torch screened through a red filter was used. After a series of trials and preliminary experiments, further extension of which is now in progress, it was found that: (1) roots grown in humid air over wet filter paper grow better than those grown under aerated water; (2) although the roots grown on wet filter paper show regular growth, their growth rate is below that of roots of similar age grown in sawdust.

It was felt necessary, therefore, to establish a standard of optimum growth rate, and then to develop a technique which could support the growth of the roots during the experiments at this level. After many trials, roots grown in sawdust, well washed and sterilized, from seed that had had 24 hours' soaking before planting were regarded as a standard of optimum growth.

Apparatus (draining-pad technique). Essentially the apparatus consisted of perspex boxes, 20×20×5 cm. with detachable fronts and containing a thin block of wood covered with cotton-wool and over this flannel and finally a layer of black cloth. A seed holder was mounted just above this padded wood block and seeds were wedged in with bits of sponge. The pad (and the atmosphere in the box) were kept saturated by a stream of distilled water or solution which dripped on to it at a rate of 300 ml./hour from a reservoir; drainage water flowed from a trough underneath.

The whole apparatus could be turned through any desired angle up to 180° without interrupting the flow of water over the pad. Each unit could hold 10 beans or larger numbers of smaller seeds.

For the run of an experiment, it was customary to soak the seeds in aerated water for 18–20 hours, then grow them in sawdust until they were about 50 hours old. The seedlings were then picked out; those with roots 10–12 mm long were set aside for the experiment. Seeds ten times as many as the seedlings required had to be soaked to allow a broad margin for selection. Selected seedlings were then rinsed in water and placed on the pads in such a manner that the seed rested inside the chamber of the seed holder and the root stretched vertically downward on the surface of the pad. The seedlings were then left undisturbed for about 15 hours to become adjusted to the new conditions before the experiment proper was performed.

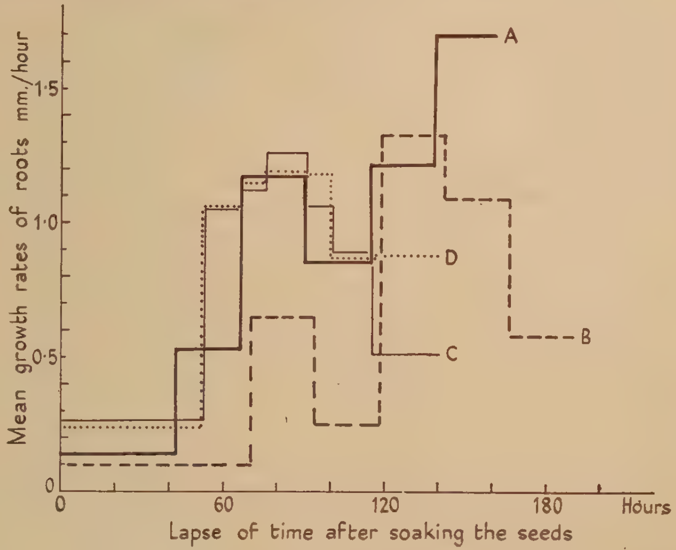

FIG. 1. Growth rates: (A) Sawdust after 24 hours soaking; (B) Sawdust after 48 hours soaking; (C) Pads with water after 18 hours soaking; (D) As (C) but with White's nutrient solution.

Measurements of root lengths and photographic recording of readings. The length of the entire root was measured every time its growth was recorded. To mark the root-base small drops of indian ink were placed by a soft brush as near as possible to the cotyledons: or a black thread was stretched in front of and across the roots at this region. The distance between the mark and the root-tip was measured with a cathetometer. Alternatively, photographs of the entire set up were made and the measurements of the roots obtained by projecting the photograph on a rigid screen using a standardized measuring wheel. The magnification was determined through the measurement of the images of millimetre scales fixed and photographed beside the roots. To avoid exposing the seedlings to white light, infra-red plates (Ilford's process and backed plates) were used in conjunction with red light supplied by four 60-watt lamps covered with red glass hoods; exposure time was 60–90 seconds with *f* 16–22.

EXPERIMENTAL RESULTS

Growth rates in sawdust and on pads. Beans soaked in water and then transferred to pads were measured as described. Results are shown in Fig. 1. The approximate constancy of growth rate between the 50th and 100th hour after soaking will be noted except for those seeds soaked for long periods

(48 hours). Growth rate fell off after 100 hours, but could not be conveniently measured in the apparatus after 120–40 hours as the roots were then too long.

Effects of decapitation. A small portion of a safety-razor blade mounted on a handle was used for decapitation. Care was taken to make the cut at right angles to the long axis of the root. The length of the removed portion of the root-tip was adjusted to requirements by watching the operation with a cathetometer through an eyepiece with a standardized ocular micrometer; or by slipping a strip of graph-paper along the side of the root-tip if the length was not less than 1 mm.

In Expts. 1, 2, and 3 the exposed surfaces of the stump were covered with lanolin paste; tips of intact control roots were similarly treated. The roots were measured with the cathetometer.

Experiment 1: Effect of removing 0·5 mm. of the tip of Vicia faba *root on growth.* Six roots were left intact and 9 roots were decapitated. The results are given in Table I. The rates of extension of the intact roots and of the decapitated roots were almost identical throughout the experiment.

It can be seen that the total growth in the 50 hours after decapitation is approximately equal to that of the control series, and it can be concluded that decapitation at 0·5 mm. behind the apex did not affect the growth of *Vicia faba* roots.

Experiment 2: Effect of removing 1 mm. of the tip. In this experiment 5 roots were left intact and 8 roots were decapitated at 1 mm. behind the apex. The results are given in Table I. Here the rate of extension of the decapitated roots was not significantly different from the control intact roots.

Experiment 3: Effect of removing 2 mm. of the tip. Six roots were left intact and 8 were decapitated at 2 mm. behind the apex. The results are given in Table I. Soon after decapitation the rate of extension of the roots dropped significantly below that of normal intact roots. This lower rate of extension continued throughout the 44 hours of the experiment. The decapitated roots were still capable of adding some 12 mm. to their length during the 44 hours following decapitation; almost all the increase took place during the first 20 hours of this period. This net increase in the length of the decapitated roots is roughly half that of the corresponding increase in length of the intact roots.

It could be safely concluded, therefore, that *V. faba* roots decapitated at 2 mm. behind the apex exhibit no acceleration in growth but, on the contrary, a significant retardation.

It was noticed, as has been repeatedly reported by earlier investigators, that the decapitated roots of this experiment showed an obvious increase in thickness. Furthermore, the stumps developed a dense coat of root-hairs. These features were not noticed in the two previous experiments when less than 2 mm. of the tip was removed.

In the three experiments just described, the exposed end of the stump was covered with lanolin to prevent free absorption of water through the cut surface; other experiments were also performed in which the exposed surface was not covered with lanolin and identical results were again obtained.

TABLE I

Effect of removing 0·5, 1·0, and 2·0 mm. of the tip of Vicia faba *root on growth*

Lapse of time (hours) after		Average growth rate (mm./hour)		Average net increase in length (mm.)		Standard error of the mean growth rate	
soaking	decap.	intact roots	decap. roots	intact roots	decap. roots	intact roots	decap. roots
0·5 mm. removed							
0	—	0·26	0·26				
50·5	—	1·19	0·97				
66·5	—	1·13	0·99				
69·5	—						
70	0	—	Decap.				
70·5	0·5	0·84	0·88	0	0	0·11	0·07
71	1	0·99	0·76	0·43	0·44	0·13	0·16
71·5	1·5	1·00	0·92	0·92	0·82	0·14	0·11
72	2	1·22	1·14	1·42	1·28	0·11	0·11
73	3	0·95	1·10	2·64	2·42	0·1	0·08
74	4	1·16	1·11	3·59	3·52	0·12	0·11
75	5	1·28	1·17	4·76	4·63	0·06	0·03
90·5	20·5	1·07	0·88	23·93	22·20	0·12	0·09
102	32	0·65	0·72	36·21	32·27	0·13	0·16
121	51			48·53	45·85		
1·0 mm. removed							
0	—	0·27	0·27				
52·5	—	1·07	0·99				
66·5	—	0·95	0·95				
69·5	—						
70	0	—	Decap.				
70·25	0·25	0·76	0·83	0	0	0·21	0·12
70·75	0·75	1·03	0·70	0·38	0·42	0·27	0·15
71·25	1·25	1·05	1·05	0·90	0·77	0·26	0·17
72·25	2·25	0·91	1·08	1·95	1·81	0·18	0·15
73·5	3·5	1·02	1·18	3·09	3·17	0·21	0·13
74·5	4·5	0·96	0·98	4·11	4·35	0·1	0·17
75·5	5·5	0·96	0·90	5·07	5·33	0·16	0·09
90·5	20·5	0·90	0·81	21·59	18·57	0·19	0·08
97·5	27·5			27·88	24·25		
2·0 mm. removed							
0	—	0·30	0·29				
51	—	1·15	1·14				
66·75	—	1·21	1·09				
69·5	—						
70	0	—	Decap.				
70·5	0·5	1·37	0·76	0	0	0·2	0·15
71	1	1·23	0·56	0·69	0·38	0·24	0·07
71·5	1·5	1·11	0·63	1·30	0·68	0·2	0·15
72	2	1·24	0·78	1·86	0·97	0·17	0·14
73	3	1·38	0·98	3·09	1·75	0·16	0·19
74	4	1·32	0·91	4·47	2·73	0·18	0·14
75	5	1·18	0·48	5·79	3·51	0·11	0·08
90·5	20·5	0·79	0·12	23·52	11·29	0·13	0·04
99·5	29·5	0·77	0·01	30·23	12·58	0·09	0·01
114·5	44·5			42·23	12·78		

Experiment 4: Effect of decapitation and of reheading on the growth of V. faba *roots when 1 mm. of the tip is removed.* Seven roots were left intact, 7 were decapitated at 1 mm. behind the apex and reheaded again with their own tips, and 6 others were similarly decapitated but not reheaded. The readings were recorded by the photographic method, and the results are given in Table II.

TABLE II

Effects of decapitation and of reheading on the growth of Vicia faba *roots when 1 or 2 mm. of the tip is removed*

Lapse of time (hours) after		Average growth rates (mm./hour)			Average net increase in length (mm.)			Standard error of mean growth rate of		
soaking	decap.	intact roots	decap. roots	rehead. roots	intact roots	decap. roots	rehead. roots	intact roots	decap. roots	rehead. roots
1 mm. removed										
0	—									
52·25	—									
67	—	0·7	0·7	0·7						
70	—	0·6	0·7	0·6						
70·25	—	—	Decap.	Decap./ rehead.	—	Decap.	Decap. rehead.			
70·75	0·25				0	0	0			
71·75	1·25	0·4	0·55	0·4	0·4	0·55	0·4	0·06	0·13	0·12
72·75	2·25	0·6	0·5	0·4	1·0	1·1	0·85	0·2	0·12	0·14
73·75	3·25	0·4	0·6	0·9	1·4	1·6	1·7	0·12	0·12	0·11
74·75	4·25	0·6	0·6	0·9	2·1	2·2	2·6	0·11	0·13	0·16
75·75	5·25	0·6	0·6	0·7	2·7	2·8	3·35	0·09	0·16	0·15
76·75	6·25	0·6	0·5	0·7	3·25	3·4	4·0	0·14	0·12	0·11
91·75	21·25	0·6	0·5	0·7	12·5	11·1	14·4	0·07	0·07	0·08
100·75	30·25	0·6	0·4	0·4	17·5	13·7	17·8	0·09	0·08	0·05
2 mm. removed										
0	—									
53	—									
67	—	0·8	0·8	0·9						
70	—	0·8	0·9	0·8						
70·25	—	—	Decap.	Decap./ rehead.	—	Decap.	Decap./ rehead.			
71	0·5				0	0	0			
72	1·5	0·8	0·5	0·5	0·8	0·5	0·5	0·12	0·09	0·11
73	2·5	0·7	0·5	0·6	1·6	1·0	1·1	0·13	0·11	0·08
74	3·5	0·8	0·7	0·5	2·3	1·7	1·6	0·1	0·1	0·1
75	4·5	0·8	0·7	0·7	3·2	2·4	2·3	0·17	0·1	0·07
76	5·5	0·9	0·6	0·6	4·1	3·0	2·9	0·17	0·11	0·12
77	6·5	0·7	0·6	0·5	4·8	3·6	3·4	0·11	0·11	0·09
91	20·5	0·6	0·3	0·3	14·0	7·4	7·9	0·08	0·05	0·06
98	27·5	0·4	0·0	0·1	17·0	7·4	8·5	0·09	0	0·04

A comparison of the rates of extension of the reheaded and of the decapitated roots reveals that, except on two occasions, there is no significant difference between the two treatments. On the two occasions where there is a significant difference, it is in terms of a rate of growth of reheaded roots slightly higher than that of the decapitated roots. In no instance did the reheaded roots grow at a rate below that of the decapitated roots.

Once more the results of this experiment confirm the results of Expt. 2 in that *V. faba* roots decapitated at 1 mm. behind the apex show a growth curve and rates of extension quite identical to those of the intact roots.

It would be justifiable, therefore, to conclude that decapitation as well as reheading of *V. faba* roots, with the cut made at 1 mm. behind the apex, did not significantly affect the rate of growth of the root.

Experiment 5: Effect of decapitation and of reheading on the growth of V. faba *roots when 2 mm. of the tip is removed.* Several experiments were performed for this purpose; but it was noticed that it was rather difficult to keep the tip stuck in place on the stump for a long period of time, first because of the relatively large size of the tip, but mainly because in a few hours the stump outgrew the tip in diameter, thus weakening the contact between the surfaces. This difficulty was overcome when the tip was stuck on the stump with a small drop of melted agar solution. This procedure was used in the experiment the results of which are described here, but it might be of interest to mention that other experiments, in which no agar was used and the tips remained on the stumps long enough, gave results identical to those described here.

Seven intact, 7 reheaded, and 7 decapitated roots were used in this experiment. The tips were stuck on their stumps with agar as mentioned above, and the exposed ends of the decapitated roots received drops of agar to insure homogeneity of the treatment. The results are given in Table II. The results show that the reheaded and the decapitated roots extend at identical rates throughout the duration of the experiment following decapitation. In other words, the replacement of the root-tip on the stump did not induce any retardation of growth as suggested by the growth-hormone theory.

The results of this experiment confirm the results of Expt. 3 in that roots of *V. faba* decapitated at 2 mm. behind the apex extended less than the intact roots. Within 20 hours following decapitation, growth stopped almost completely, and the increase in length gained by the decapitated roots during this period was almost half the corresponding increase in length gained by the intact roots.

DISCUSSION

Roots which had been subjected to these various treatments were fixed and sections were cut and the structure compared with control untreated roots. Dimensions were found to be approximately as follows: root-cap 0·5–0·7 mm., meristem 0·5–0·6 mm. Thus 0·5 mm. decapitation removes cap only and 1·0 mm. decapitation also removed at least half or even more of the meristem. The whole meristem is removed by 2·0 mm. decapitation.

Two days after decapitation, those roots which had 0·5 mm. removed had recovered, and a new root-cap had been developed so that the structure was virtually identical with control roots. No regeneration of meristem occurred when 2·0 mm. had been removed.

Effects of these decapitation treatments on the normal positive geotropic responses of the roots are discussed in Part II.

It seems clear from the results of Tables I and II that decapitation of up to 1·0 mm. has not greatly affected growth rates, but, as cell counts were not made in these experiments, relative numbers of meristematic and vacuolating cells are not known.

Under the conditions of these experiments in which decapitated, reheaded, and control roots were all grown in their normal vertical orientation, there is no significant difference between the three treatments, and one must conclude that although the presence of the root-cap is essential for effective production of a geotropic response, there is no adequate evidence that a root-growth retarding substance is produced by the root-cap when it is left in its normal vertical position.

PART II. EFFECTS OF DECAPITATION AND OF REHEADING ON THE GEOTROPISM OF *VICIA FABA* ROOTS

SUMMARY

Roots of *V. faba* decapitated at 0·5 mm. from the tip, and roots decapitated in the same manner, then reheaded with their own tips, respond to gravity when placed horizontal in exactly the same manner as the intact control roots.

Roots decapitated at 1 or 2 mm. behind the apex do not respond to the stimulus of gravity. Similarly, roots decapitated at 1 or 2 mm., then reheaded with their own tips, do not respond to gravity.

The geotropic curvature of the root is due to an increase in the length of the cells on the upper side and not due to an increase in their number.

The role of the root-tip in the geotropic irritability of the entire organ was recognized long ago by such investigators as Ciesielski (1872) and Darwin (1880). Experiments of these investigators as well as the experiments of Pfeffer (1894), Snow (1923), Cholodny (1924, 1926), Keeble, Nelson, and Snow (1929), and Hawker (1932) all lead to the conclusion that decapitation of the root-tip renders the root insensitive to gravity. On the other hand, Sachs (1882) objected to Darwin's experiments and hence to his conclusions. Gorter (1932) showed that decapitated roots are as responsive to gravity as the intact roots; while Czaja (1935) showed that decapitated roots respond negatively to gravity. Snow's experiments (1923) added that, if the tips of decapitated roots are stuck on again with gelatine, the roots in most cases become again capable of bending down in response to gravity. This finding was supported by the experiments of Cholodny (1924, 1926), who added that reheading with a root-tip or with a coleoptile tip restores the geotropic sensitivity to the decapitated root. His rather important conclusion was that the root-tip, like the coleoptile tip, is a seat of growth-substance formation.

Experiments of other investigators, as Naundorf (1940), support the finding that the root-tip is the receptive part of the root in tropic reactions. So, with the exception of Gorter (1932) and Czaja (1935), there is a common agreement, which is even acknowledged in standard textbooks, that decapitation checks the sensitivity of the root and that reheading restores this character to them.

In spite of considerable lack of agreement to be noted in the earlier investigations (Cholodny, Gorter, *loc. cit.*) important conclusions have been drawn, and these are often based on work lacking two important features:

(*a*) standardized homogeneous conditions for growing the roots during the experiment, and (*b*) proper statistical treatment of the results. It was felt desirable, therefore, to reinvestigate the role of the root-tip in the geotropism after paying due consideration to these two points.

Material and technique. Vicia faba was used in the present investigation. Details of the procedure and apparatus used are given in Part I. Briefly, the draining-pad technique was employed, and the readings were recorded photographically, using red light and infra-red plates. The seeds were soaked for 18 hours, sown in sawdust until they were about 50 hours old, then transferred to the apparatus. Decapitation was performed when the roots were 70 hours old. The operation was done with a safety-razor blade and the tips stuck back without use of an adhesive. Other experiments in which 1 per cent. agar was used to stick the tip to the stump were also performed; they are not described in the present study since they gave the same results. Stimulation was effected soon after the operation by turning the apparatus 90° to vertical, i.e. placing the roots horizontal. The curvatures of the root-tips were measured on the projected images of the roots with reference to the vertical plane indicated by a thread hung along the side of the apparatus. A protractor was especially constructed to be used for the measurement of the curvatures of the root-tips. It is a half-circle, diameter 20 cm., of thin perspex, with an arm of the same perspex 40 cm. long and 3 cm. wide mounted at one end to turn round the centre of the protractor. Two pairs of lines are drawn on the arm parallel to and on either side of the indicator line radiating from the centre of the protractor. All the lines are 3 mm. apart. To measure the curvature of a root-tip, the base line of the protractor is laid on the projected image of the apparatus at right angles to the vertical plane, then the image of the root-tip is symmetrically enclosed between any two parallel lines of the arm. The indicator line on the arm of the protractor then reads an angle on the scale which corresponds to the deviation of the root-tip from the vertical plane.

EXPERIMENTAL RESULTS

It was shown in Part I that in *V. faba* roots the root-cap occupies the terminal 0·5–0·7 mm. of the tip; then follows the primary meristem which occupies another region of 0·5–0·6 mm. In the three experiments described below, the decapitation is made at 0·5, 1, and 2 mm. from the apex to study the effect of removing the different regions of the tip on the geotropism of the root.

In Expt. 1, a variety of *V. faba* called 'Baladi Rebayah 34' obtained from the Plant Breeding Department, Ministry of Agriculture, Cairo, Egypt, was used. In the other two experiments, another variety called 'Windsor' obtained from a seed merchant in London was used. The two varieties differ in some features, in that the 'Baladi' seeds are smaller and produce roots which are thinner than those of the 'Windsor' variety. Otherwise the growth curves of roots of both varieties are virtually identical.

Experiment 1: Effect of removing 0·5 mm. of the tip on the geotropism of the root. Nine intact, 10 decapitated, and 10 reheaded roots were used in this experiment. Since it is difficult to remove precisely 0·5 mm. of the tip, care was taken to remove the root-cap only, the location of which is quite easy when a magnifying lens and a weak spot-light are used. The root-cap, as stated before, does not exceed 0·7 mm. in length. The mean curvatures of the root tips of the three groups of roots are given in Fig. 1 *a*.

It is obvious from the figure that the three groups of roots respond to the stimulus of gravity in the same manner. Nevertheless, a statistical study of the data was made to detect whether the slight differences thus recorded are of any significance. The *t*-test of significance was applied in the usual way. At $P = 0{\cdot}05$ it was found that all the differences noticed between the three treatments are statistically non-significant. This means that the presence or absence of the terminal 0·5 mm. of the root-tip of *V. faba*, i.e. the root-cap, has no effect on the ability of the root to respond to gravity by a positive geotropic curvature.

Experiment 2: Effect of removing 1 mm. of the tip. Seven roots were left intact, 10 were decapitated, and 9 reheaded with their own tips; but 4 of the latter had their tips replaced inefficiently and, therefore, the means of the remaining 5 are given here. The results are also shown in Fig. 1 *b*.

It is obvious from the figure that the intact roots respond to gravity by a characteristic downward bend soon after being placed horizontal. On the other hand, the decapitated and the reheaded roots as well remain almost horizontal throughout the experiment. The figure also shows that the reheaded roots are curved downward more than the decapitated roots; thus, after 4 hours of being placed horizontal, the reheaded roots show an average downward curvature of 11° bigger than that of the decapitated roots. All the differences thus recorded between the decapitated and the reheaded roots, including the big value of 11°, are statistically non-significant. The results of this experiment, therefore, show clearly that decapitation at 1 mm. behind the apex checks the ability of the *V. faba* roots to respond to gravity, and that reheading does not restore that lost feature.

Experiment 3: Effect of removing 2 mm. of the root-tip. Seven intact, 8 decapitated, and 9 reheaded roots were used in this experiment. The results are given in Fig. 1 *c*.

As in the previous experiment, the figure shows clearly that the decapitated and the reheaded roots do not show any tendency to bend downward when placed horizontal. The intact roots, on the other hand, react to the stimulus of gravity within 30 minutes of being placed horizontal. Such results show that decapitation at 2 mm. affects response to gravity in the same way as 1 mm. decapitation.

Internal features of the geotropically curved root

Since actively growing roots bend downward when placed horizontal, there must be unequal growth of the two sides. The literature affords little certain information on whether this unequal expansion is due to unequal frequency

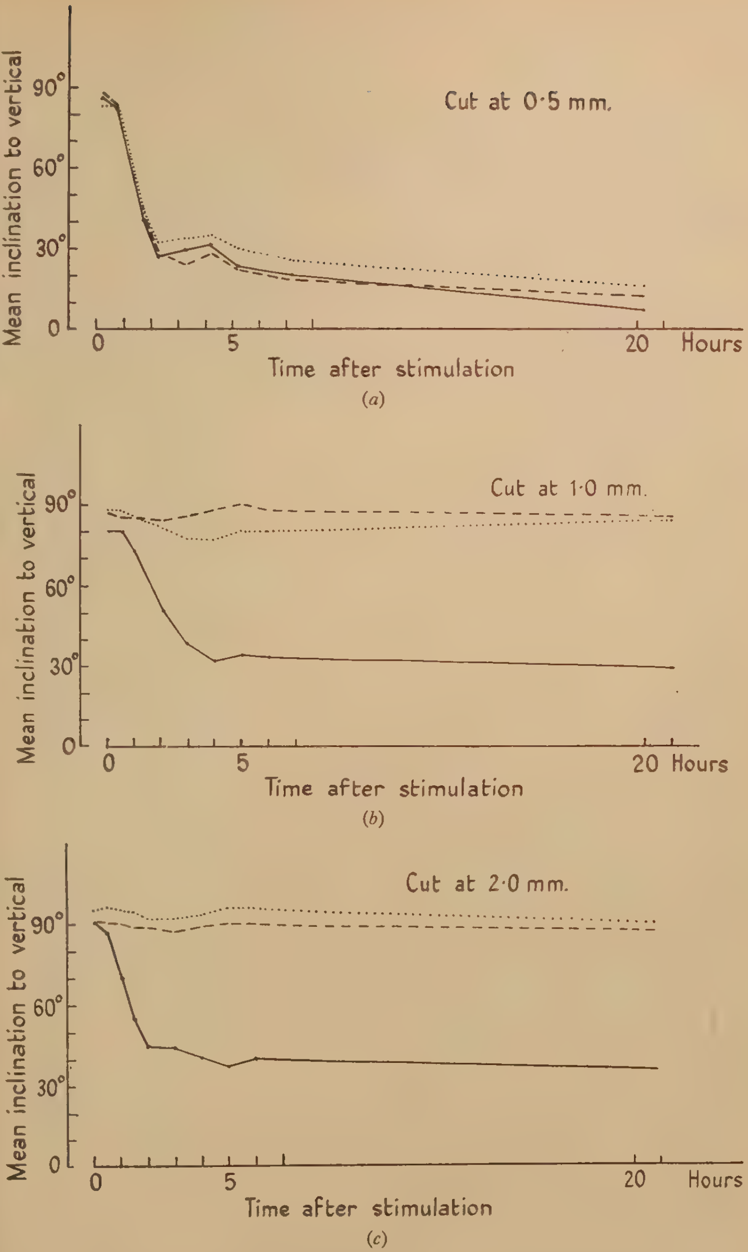

FIG. 1. Inclination after stimulation. Intact roots (——); decapitated (– – –); decapitated and reheaded (········). Means of 10 to 5 roots, see text.

of mitosis or to unequal growth of the cells. Thus Frank (1868) noticed that no cell division accompanied the geotropic curvature of roots and that the cells are longer on the convex than on the concave side. Wagner (1936), on the other hand, conducted a detailed study of the mitosis in the geotropically stimulated roots and found that the geotropic curvature is accompanied by an increase in mitosis on the side becoming convex. Since the decapitation at 1 and 2 mm. is bound to remove a part of the meristem, and hence interfere with mitosis, it is quite important to determine whether the geotropic curvature is due to change in the size of cells as found by Frank or to change in mitotic activity as found by Wagner.

Seedlings of *Sinapis alba* and *Vicia faba* were germinated and grown on the wet pads in the usual manner described before; then they were stimulated by gravity at 135° to vertical. At regular time-intervals following stimulation, the curving root-tips, about 10 mm. long, were cut off and fixed in alcohol-formaline, dehydrated, then embedded in paraffin wax and sectioned longitudinally (10–12 μ) and stained with Chloroazol Black E (procedure after Conn and Darrow, 1946). Photomicrographs of the selected sections were made. A series of enlargements were made for the different parts of the radial longitudinal section chosen. Aided by marks laid beforehand on the coverslip of the preparation, the prints were assembled together to reconstruct an enlarged photograph of the entire root section. Counts and measures of cells were made on such photographs.

In *Sinapis alba* and in *Vicia faba* it was found that the geotropic curvature of the root is due to an increase in the length of the cells on the convex side and not to an increase in the number of cells on this side.

DISCUSSION

Experiment 1 shows clearly that the terminal 0·5 mm. of the root-tip of *V. faba* is in no way essential in directing or controlling the geotropic irritability of the organ. The root cap of *V. faba* is apparently therefore not a source of growth hormone, if there is any growth hormone in the root-tip. Similarly, it seems that the statolith theory developed mainly by Nemec (1900, 1902) and Haberlandt (1903) in which the starch grains in the root-cap are assumed to play a role in the perception of geotropic stimulus is not supported by the results of this experiment.

Experiments 2 and 3 show that (*a*) roots decapitated behind a short or a long region of the primary meristem lose their ability to respond to gravity, and (*b*) replacement of the tip on the stump does not restore the lost feature. The first conclusion is in accord with most of the early investigations, but the second conclusion is not.

Experiments of Snow (1923) were conducted on *Vicia faba*, which is also the material used in the present investigation. But his conclusion was that roots decapitated at 2 mm. and reheaded with their tips were, in most cases, capable of bending down in response to gravity, a result which I failed to prove. The few differences between Snow's procedure and the procedure which I followed cannot account for the different results obtained. It may be of interest to notice that in Snow's experiments the control intact roots when laid horizontal showed a curvature of not more than 45° in 15 to 24 hours, while corresponding roots in my experiments showed a curvature of this angle in less than 3 hours and a curvature of 55° to 60° in about 20 hours.

That the root-tip, like the grass coleoptile, is a source of growth substance is the conclusion which Cholodny derived from a series of investigations in which experiments unlike those of Snow were performed. Thus in 1924 he found that roots of *Zea mays* and of *Lupinus angustifolius* when decapitated at 1 to 1·5 mm., then reheaded with *Zea mays* coleoptile tips, curved quite normally, whereas *Zea mays* root-stumps reheaded with *Avena sativa* coleoptile tips did not curve at all. In 1926 and 1928 he completed the story by showing that *Lupinus angustifolius* hypocotyl segments from which the central cylinder was removed and a *Zea mays* root-tip introduced into the hollow cylinder (1926) responded normally (negatively); and decapitated *Avena* coleoptiles reheaded with *Zea mays* root-tips (1928) responded normally to geotropic stimuli, again showing negative geotropism. Such experiments point indirectly to the presence of a growth substance in the root-tip, but do not afford an answer to whether or not a root-stump reheaded by its own tip can respond to gravity.

Gorter (1932) claims that *Pisum sativum* roots decapitated at 1 mm. curve in response to gravity in the same way as the intact roots, and that decapitated *Avena* coleoptiles fail to respond to gravity when they are reheaded by root-tips. Cholodny (1933) replied to Gorter's claims that the conditions under which she conducted her experiments were rather dry and that she was not precise in making the decapitation. The clear difference between the results of Expts. 1 and 2 of the present investigation shows that it is of prime importance to be precise in placing the cut.

Keeble, Nelson, and Snow (1929) verified the argument that the presence of a growth substance in the root-tip and its diffusion backward is responsible for the geotropic reaction in the organ by the following experiments. Using *Zea mays* seedlings, they found that: (1) When unstimulated root-stumps were reheaded by tips from roots which had been placed horizontal for 1 to $1\frac{1}{2}$ hours, 11·8 per cent. of the reconstructed roots curved within 24 hours towards the side of the tip that had been lowest. (2) When root-stumps were placed horizontal for 2 hours, then placed vertical and reheaded by tips of unstimulated roots, 10·5 per cent. of the reconstructed roots curved within 24 hours towards the side of the stump that had been lowest when horizontal. In conformity with the Cholodny–Went theory, Keeble *et al.* interpret their results in the following way: From a geotropically stimulated tip the growth substance diffuses out in unequal concentrations on the two sides, and so causes an unstimulated stump to curve. From an unstimulated tip it diffuses out on all sides equally, but on passing into a stump which has been geotropically stimulated it is there somehow redistributed so as to reach the upper and lower sides in different concentrations.

It is obvious that the results of the first type of experiments of Keeble *et al.* are in direct disagreement with the results of the experiments described in the present investigation. A careful study of the procedure followed by these investigators and of the points upon which they based their conclusions throws considerable doubt on the value of these conclusions. It seems likely that the period of 1 to 2 hours of stimulation by gravity of the tip, followed by a period of 24 hours at normal vertical position, would result in counteraction of the effect of the stimulus. Any observed curvature of 10 or 12 per cent. of the roots whilst 88 or 90 per cent. fail to show a positive curvature suggests that autonomic movements are being observed. The authors themselves state that the results of the second type of their experiments were non-significant, and the significance of the first group seems doubtful.

The experiments described by Hawker (1932) are in a way similar to those of Keeble *et al.* Hawker divided her *Vicia faba* roots into four groups: (*a*) Decapitated roots laid horizontal for 3 to 4 hours then reheaded with tips cut from intact roots that have been stimulated for 3 to 4 hours also in a horizontal position. The roots were reconstructed in such a way that the under side of the tip was in line with the upper side of the stump. (*b*) Similar to (*a*) except that the under side of the tip was in line with the under side of the stump. (*c*) Roots laid horizontal for 3 to 4 hours, then decapitated. (*d*) Roots laid horizontal for 3 to 4 hours, then left intact. After this period of *3–4 hours* all four groups of roots were placed in a vertical position. After *24 hours* the curvatures of the reconstructed roots, of the stumps, and of the intact roots were recorded. Hawker found that roots of group (*a*) and group (*b*), like those of the control groups (*c*) and (*d*), curved towards the side of the tip which was lowermost, thus leading her to conclude that the root-tip has a stronger directional influence on response to gravity than has the root-stump.

From the experiments described in the present investigation, it is easily noticeable that intact *Vicia faba* roots when laid horizontal start to curve downwards within 30 minutes and show an average curvature of about 50° within 2 to 4 hours. Such a big curvature could not have escaped Hawker's observation. One wonders, therefore, whether roots of groups (*c*) and (*d*) in her experiments were already curved before they were placed vertical! If her roots did not curve downwards during this 3 to 4 hours' period, something should account for their abnormal behaviour. And even if they did curve normally, then when they were placed in a vertical position the roots would have received an opposite stimulus, the duration of which, 24 hours, should be long enough to cause the roots of group (*d*) at least to become straight again. But this is not the case in Hawker's results since, according to her figures, 70 per cent. of the roots of this group were still curved towards the side that was lowermost 24 hours after these intact roots were placed in the vertical position.

To describe the curvatures of groups (*a*) and (*b*) in her experiments, Hawker assumed that the tips and stumps of the reconstructed roots contained, as a result of being pre-stimulated by gravity at a horizontal position, a growth substance the concentration of which is higher in the side that was underneath than in the opposite side. This assumption is open to the same criticism as that stated above, namely that the curvatures of the root-tips were recorded 24 hours after the roots were placed in the vertical position, so that if 3 to 4 hours in a horizontal position is long enough to induce a stimulus, then 24 hours in a vertical position is sure to induce an opposite stimulus and cause the root to become straight again.

In experiments with roots, autonomic curvatures are frequently observed, and unless such curvatures are allowed for by appropriate statistical analysis, it would be difficult to decide by simple comparison of the observations whether a curvature is genuine or autonomic. Further, the method of photographic recording of the readings used in the present investigation made it possible to follow the reaction at short intervals. The main feature of the geotropic reaction thus revealed is the fact that most of the curvature occurs during the first 3 hours following stimulation. The value of the early investigations in this field is, therefore, very limited, since in most cases the readings were recorded at very long time-intervals.

From the discussion above, it is obvious that the argument in favour of a growth substance being formed in the root-tip and responsible for inducing a curvature in the zone of elongation of the root as a result of geotropic stimulation is not strongly supported.

The fact that decapitated roots of *V. faba* did not curve downwards when placed horizontal stresses the assumption that the root-tip plays an important role in the chain of reactions leading to the geotropic curvature. It is possible, therefore, that the tip produces a substance in the manner suggested by the Cholodny–Went theory; and it is necessary to find an explanation for the failure of the tip to carry out its function when the root is reheaded, and the role of the tip may therefore be much more complex than the Cholodny–Went hypothesis suggests.

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Studies on Plant Growth Hormones

III. APPLICATION OF ENZYME REACTION KINETICS TO CELL ELONGATION IN THE *AVENA* COLEOPTILE

S. HOUSLEY, J. A. BENTLEY, AND A. S. BICKLE
Department of Botany, Manchester University

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SUMMARY

1. Previous reports that Michaelis enzyme kinetics may be applied to the system controlling cell elongation in *Avena* coleoptiles are confirmed.

2. Substrate (applied hormone) and enzyme are not in direct contact with each other. Active transport of the applied hormone in coleoptile sections, and permeability of cells to hormone, regulate the intracellular substrate concentration, thereby markedly influencing the value of K_s, a parameter giving a measure of the affinity of substrate for enzyme.

3. The effect of permeability and transport factors on calculating K_I values of competitive hormone inhibitors is considered. Data of other workers are used to show that observed K_I values are not necessarily independent of the hormone used.

4. Auxin-induced inhibition of cell elongation results primarily from a toxic action of hormones on cell protoplasm leading finally to cellular disorganization, shrinkage, and death. The data do not decisively support the hypothesis of inhibition resulting from steric hindrance to two-point attachment between substrate and enzyme.

INTRODUCTION

In three recent papers (Foster, McRae, and Bonner, 1952; McRae and Bonner, 1953; and McRae, Foster, and Bonner, 1953) it is claimed that the elongation of *Avena* coleoptile sections, as controlled by applied auxins and anti-auxins, may be considered in terms of the enzyme kinetics of Michaelis and Menten (1913) and those subsequently formulated by Lineweaver and Burk (1934). It is also claimed that not only do physiological concentrations of hormone follow these kinetics, but by their extension to include two-point attachment between hormone (substrate) and enzyme (the literature is summarized by Muir and Hansch, 1953), growth inhibitions resulting from supra-optimal concentrations of auxin may be fully accounted for.

Avena data obtained in this laboratory do not support all these conclusions of Bonner and co-workers. Therefore in this present paper Michaelis kinetic treatment is applied to the growth of *Avena* coleoptile sections in solutions of low and high hormone concentrations so that the American claims can be critically examined.

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METHODS

The method of *Avena* straight-growth assay has already been described (Bentley, 1950) and the techniques reported in detail (Bentley and Housley, 1954). In all experiments coleoptiles are selected for uniform length over a 2-mm. range: a 10-mm. section is cut 3 mm. from the tip of each coleoptile. Ten section replicates per treatment were used except when stated to the contrary. All growth-curve experiments were carried out at 27° C.; the remaining experiments were at 25° C.

Throughout this paper the kinetic symbolism adopted by McRae and Bonner (1953) has been used.

KINETIC TREATMENT OF GROWTH AT LOW HORMONE CONCENTRATIONS

Michaelis kinetics account for reactions of a system in which enzyme and substrate are in direct contact with each other. Bonner and co-workers have applied these kinetics to cell elongation of the *Avena* coleoptile, a system in which enzyme or receptor entity is separated from applied substrate (i.e. hormone) by appreciable physical and or physiological barriers. Furthermore, they consider the parameters of the kinetics to be constants which truly and solely reflect the properties of enzyme-substrate attachment.

Two factors affecting availability of applied hormone to receptor entity in the *Avena* coleoptile are permeability of cell membranes and transport of applied hormone within the tissues. Transport can be studied by blocking either or both ends of coleoptile sections with petroleum jelly and then floating on hormone solution. It was considered that a kinetic analysis of blocked and non-blocked sections would provide a possible method of deciding whether such an analytical treatment of cell elongation in a complex organ is justified. An examination of this point is made in Expt. B244.

A method of testing the effect of permeability is to make a kinetic analysis of growth curves obtained with 3-indolylacetonitrile (IAN), 3-indolylacetic acid (IAA), and the ethyl ester of IAA (IAEt). These molecules have widely differing degrees of chemical polarity: furthermore, the literature suggests that IAN and IAEt only show physiological activity after hydrolysis to IAA has occurred (e.g. Veldstra, 1944; Thimann, 1951 and 1953); thus, these three indole compounds will initially have different permeabilities, but subsequently will result in identical substrate-enzyme complexes. They also have the advantage of being naturally occurring and there will, therefore, probably be a minimum interference from other properties such as differing degrees of transport within the coleoptile sections. In Expt. B245 these three hormones are subjected to a kinetic analysis.

Effect of hormone transport within the tissues. The existence of hormone transport in coleoptile sections floating in auxin solution has been established in experiments in which apical, basal, and both ends of sections are blocked with petroleum jelly (B.P. standard) prior to hormone treatment. Microscopical examination shows that this treatment effectively covers the cells of the cut surface. Care was taken during experiments to prevent the epidermis of the sections from becoming contaminated with the jelly.

TABLE I

Effect on response of blocking either or both ends of coleoptile sections with petroleum jelly. Figures denote final section length (mm.) in solutions of 1·0 mg./l. IAA and in 1·0 mg./l. IAN: the corresponding standard errors are also shown. 40 section replicates per treatment were used in Expt. B231, 30 in B233, and 20 in the remaining experiments.

Expt. No.	Hormone treatment	Apex blocked	Base blocked	Both ends blocked	No blocking
B231	IAA	16·06±0·13	16·84±0·17	15·52±0·26	18·25±0·12
B233	IAA	14·57±0·22	15·21±0·22	13·87±0·24	16·43±0·12
B240	IAN	16·46±0·19	16·34±0·15	15·62±0·31	17·75±0·14
B241	IAN	17·42±0·20	17·17±0·21	15·64±0·36	18·52±0·20
B242	IAN	15·87±0·13	15·87±0·17	14·69±0·21	16·80±0·16

Results of typical experiments of 22 hours' duration are given in Table I. It is clear that the greatest response is obtained when both section ends are free, the least response when both are blocked, while apical and basal blocking causes an intermediate response. Differences between no blocking (control) and all other treatments are statistically significant at the 1 per cent. probability level (*P*). With IAA, apical blocking is more effective than basal blocking in reducing response, these treatment differences being significant (.1 per cent. *P* in B231: 5 per cent. *P* in B233). With IAN, response to apical and basal blocking is either identical or of the same order: treatment differences are not significant at 5 per cent. *P* and the variance ratio values are small. There is probably easier entry of IAN through the epidermis due to its greater effect on permeability (see later), and this effect overrides any differences due to entry through apical or basal cut surface. With IAA the greater response with basal blocking than with apical blocking suggests that there is a tendency for polarity of hormone transport to occur.

The results are not likely to be due to a possible inhibitory substance contained in the petroleum jelly, because of these differences in response, and also in the appearance of sections obtained with IAA or with IAN. Sections with apical blocking or with both ends blocked curve considerably with IAA, whereas sections basally blocked or not blocked are much straighter. This is not shown with IAN, all sections being straight. These experiments have been repeated many times. The results, therefore, do suggest that response to blocking with petroleum jelly results from interference with hormone entry into the coleoptile section, and not from the effect of some substance in the jelly.

From the results in Table I it can be concluded that an appreciable quantity of hormone used in coleoptile section growth enters through the cut ends of the section; also, experiments of more than 24 hours' duration show that hormone entry through the epidermis cannot entirely replace apical and basal entry no matter how long the sections remain in solution. Thus these experiments suggest that floating coleoptile sections on hormone solutions does not eliminate the transport factor in coleoptile growth, a conclusion contrary to previous opinion (see, for example, Veldstra, 1944; and Bentley, 1950). It will therefore be of interest to apply Michaelis kinetics to coleoptile sections with a normal hormone transport system and with a partially blocked system. This is examined in the following experiment (B244).

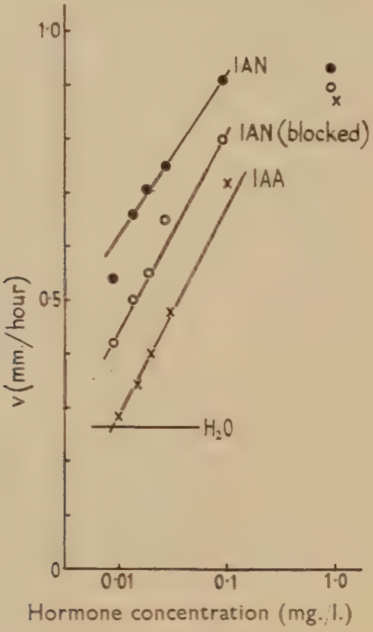

FIG. 1. (Expt. B244.) Initial velocity (*v*) of coleoptile section growth in solutions of IAA –×–, IAN –●–, and water. Sections in one set of IAN treatments –○– had the apical end blocked with petroleum jelly.

Solutions of 1·0, 0·1, 0·03, 0·02, 0·015, and 0·01 mg. l. IAA and molar equivalents of IAN were used. Growth curves were obtained of non-blocked coleoptile sections with these treatments, and of sections apically blocked with petroleum jelly with the IAN treatments. Previous work (Bentley and Housley, 1953) has shown that under the present conditions section growth does not deviate markedly from linearity with time over the first 4 hours. Initial velocities of section growth were therefore taken from curves obtained by section measurement after 2, 3, 4 and either 5 or 6 hours.

Initial velocities of section growth for the given treatments are shown (Fig. 1) plotted against hormone concentration on a logarithmic scale. The reciprocal of the initial section velocity plotted against the reciprocal of the hormone concentration is given in Fig. 2. It is seen that a linear relationship exists, that the intercepts are almost coincident on the ordinate, that the gradients differ, and of necessity intercepts on the abscissa differ too. It is of interest to calculate the latter since in Michaelis kinetics it is considered to be

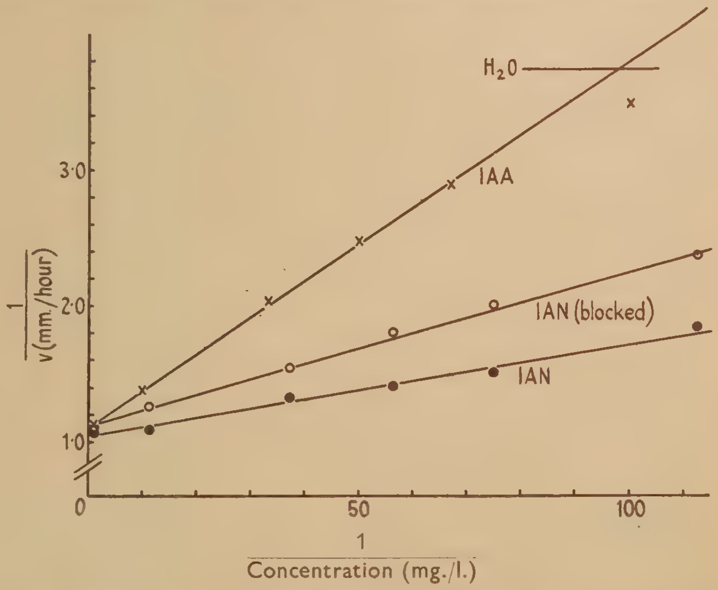

FIG. 2. (Expt. B244.) Relationship between the reciprocal of initial velocity of coleoptile section growth ($1/v$) and the reciprocal of the hormone concentration. Hormone treatments and key as for Fig. 1.

a measure of the affinity of an enzyme for its substrate. Values of the abscissa intercepts are as follows: IAN (sections not blocked) —153, IAN (sections blocked) —98·2, IAA (sections not blocked) —40·4.

Discussion on effect of transport. It is seen from Table I and from Fig. 1 that blocking of coleoptile sections with petroleum jelly reduces the initial velocity of section growth and final section length. Although the former has only been demonstrated with IAN, by analogy, results in Table I suggest that it is true for IAA too. These observations suggest that transport of externally applied hormone occurs in coleoptile sections floating in hormone solution: furthermore, this transport is an essential factor for normal section growth, as hormone entry through the epidermis cannot entirely replace it no matter how long the sections remain in solution. The effect of this transport factor is reflected in the graphs of Fig. 2. As the intercept on the abscissa is a measure of enzyme/substrate affinity, the affinity of receptor entity for IAN or its hydrolytic products (and with virtual certainty, for externally applied IAA too) appears to be influenced by hormone transport in the coleoptile.

However, the affinity of a substrate for an enzyme depends only on the nature of substrate and enzyme. Thus it is clear that in the *Avena* coleoptile one is not dealing with an *in vitro* type of enzyme system, but one in which at least one secondary factor is present (i.e. hormone transport in the coleoptile), this factor being capable of masking the true value of K_m, the parameter associated with the enzyme-substrate affinity.

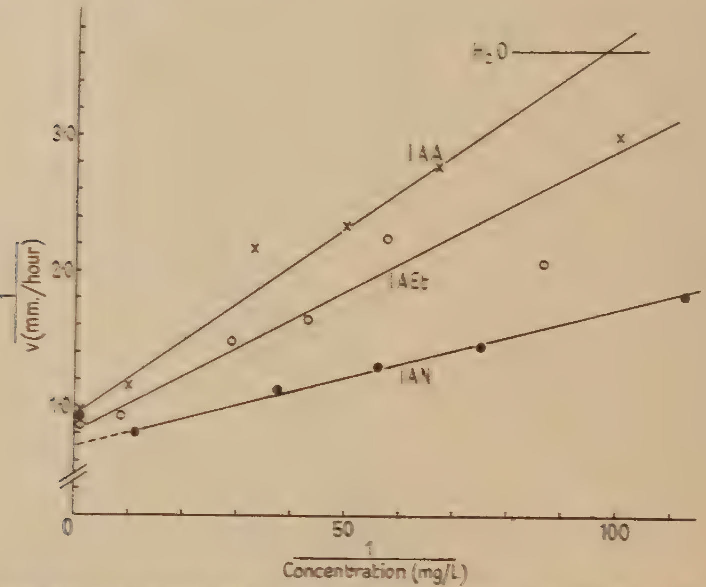

FIG. 3. (Expt. B245.) Relationship between the reciprocal of initial velocity of coleoptile section growth (1/v) and the reciprocal of the hormone concentration.

Effect of permeability of cell membranes. The growth curve experiment of coleoptile sections in IAN, IAA, and IAEt (B245) was carried out as in the previous experiment, using the same acid and nitrile concentrations and molar equivalents of IAEt. Kinetic treatment of the results is shown in Fig. 3. Again it is seen that a linear relationship holds, although the data are more scattered than in Fig. 2: the three gradients differ, but intercepts on the ordinate are not so close. Intercepts on the abscissa are as follows: IAN −71·3, IAEt −38·1, and IAA −33·8.

From the results in Figs. 2 and 3 it is concluded that a linear relationship exists between the reciprocal of the initial velocity of coleoptile section growth and the reciprocal of the hormone concentration. The present work has shown that this at least holds over the concentration range 1·0 mg./l. to the approximate lower limit of sensitivity at 10^{-4} mg./l. It would seem, therefore, that Michaelis kinetics can account for the growth of *Avena* coleoptile sections in solutions of IAN, IAA, and IAEt at sub-optimal concentrations.

Discussion on effect of permeability. Bentley and Bickle (1952) concluded that IAN is transported through *Avena* coleoptile tissues as easily as IAA: it is quite possible that the transport of IAEt is similar as it is reported to be naturally occurring (Redermann, Wittwer, and Sell, 1951). It is therefore probable that the transport factor will not markedly influence the comparative values of the abscissa intercepts in Fig. 3. If it is assumed that IAN and IAEt are consistently hydrolysed to IAA before physiological activity occurs so that growth results from an IAA/receptor entity complex, then the principal factor influencing the results is most likely to be permeability of the coleoptile cells to the three indole compounds. If this is so, then one will expect the abscissa intercept (i.e. $-1/K_s$) to reflect the permeability of the coleoptile cells to the three compounds. It is significant that intercepts on the abscissa in Fig. 3 (IAN $-71{\cdot}3$, IAEt $-38{\cdot}1$, and IAA $-33{\cdot}8$) reflect the order of increasing chemical polarity and decreasing lipoid solubility from a fairly neutral molecule (IAN) to an acidic molecule (IAA). With due regard to the assumptions made above, it is concluded that permeability of *Avena* coleoptile tissue to applied growth regulators is capable of masking the true values of the parameters associated with the abscissa intercept.

At this point it is useful to consider and apply the conclusions of the two previous sections to the kinetics of cell elongation. It has been seen that active transport in coleoptile sections and permeability of the tissues prevent the ready access of applied hormone to growth centres in the cell. It is self-evident that permeability is the more important; furthermore, the greater the permeability the less important the transport factor becomes in helping to increase the intracellular concentration. It is apparent then that unless the permeability of the tissues to a hormone is high, there will be an appreciable difference between intracellular and externally applied concentrations. The effect of this on Michaelis kinetics may be illustrated by using the IAN (sections apically blocked) and IAA graphs in Fig. 2 (assuming that they are coincident at the zero abscissa). One may suppose that the IAN record represents the hypothetical graph that would be obtained for a hormone if there was no discrepancy between intracellular and applied concentrations and that the IAA data represent external concentrations which, because of low permeability, exceed the intracellular concentrations. The intercepts on the horizontal axis of this graph give the reciprocals of the observed K_s values. Thus it is clear that the observed affinity ($1/K_s$) where external concentration exceeds internal will be smaller than the true affinity of the regulator for receptor entity, and that the observed K_s will be correspondingly larger than its true value.

Consideration of the effect of transport and permeability factors on competitive inhibition of hormone action. Although it is not possible to measure experimentally true K_s values, it should be possible to show an effect of the transport and permeability factors on observed K_s or K_I values. Data for doing this are given by McRae and Bonner (1953) in their Tables 7 and 10, but it is unfortunate that the design of their experiments is poor for illustrating this point decisively: however, this need not detract from considering the method. Their experiments consisted of examining the effect of three inhibitors, 4-chlorophenoxyisobutyric acid (4-CPIB), 2:4-dichlorophenoxyisobutyric acid (2:4-DCPIB), and 2:4-dichloroanisole (2:4-DCA) on the *Avena* straight-growth test, each inhibitor being tested in the presence of IAA and of 2:4-dichlorophenoxyacetic acid (2:4-D): a basal medium of 3 per cent. sucrose and 0·0025 M potassium maleate buffer (pH 4·5) was used. By carrying out an appropriate kinetic analysis (see McRae and Bonner, loc. cit.), they conclude that each inhibitor competes with IAA and 2:4-D for active sites on the receptor entity. K_I values for each inhibitor in the presence of IAA and of 2:4-D are given. It is pointed out that the affinity of a competitive inhibitor for the receptive site within the cells depends only on the nature of the inhibitor and of the site; therefore it follows that the K_I values should be independent of the nature of the growth-promoting regulator against which the inhibitor is tested. Now this presupposes that the intracellular concentration of the regulators are identical with the concentrations in the solution surrounding the coleoptile sections, or alternatively that the ratio of the intracellular concentrations of the growth promoters to inhibitors is identical with the ratio in the external solution. The former would yield true K_s and K_I values, the latter false ones, but the false values would be consistent and therefore one would expect the false K_I values in IAA and in 2:4-D to be equal.

Let us consider the experiments of McRae and Bonner from this aspect. It may be concluded that transport of IAA in coleoptile sections is greater than that of 2:4-D (Pincus and Thimann, 1948, p. 27). In order to simplify the analysis carried out in the following paragraph it will be assumed that the permeabilities of coleoptile tissues to IAA and 2:4-D are equal, although in fact they are probably only of the same order, that of IAA being the greater; however, the conclusion drawn from the analysis does not differ if the permeabilities are taken as being equal. Since IAA is more quickly transported, the ratio of intracellular to external concentration will be greater with IAA than 2:4-D. This will result in different K_I values with IAA and with 2:4-D for each inhibitor provided the permeabilities of inhibitor and growth promoter are independent of each other and there is no interaction between them.

Fig. 4 represents the results of an hypothetical experiment carried out with several concentrations of IAA and one concentration of inhibitor. Suppose graph *A* is the observed result for IAA with no inhibitor present. If one now imagines the transport factor of IAA to be reduced until it equals that of 2:4-D, the intracellular concentrations of IAA will become smaller, resulting in smaller values of *v*. The graph representing this hypothetical condition is shown by *B*. *C* is the observed result when the inhibitor is incorporated with IAA solutions, while *D* is the hypothetical condition of inhibitor and of IAA with reduced transport. The value of K_I can be calculated from equation (1)

$$\frac{1}{v}=\frac{1}{V_{\max}}\left[K_s+\frac{K_s[I]}{K_I}\right]\frac{1}{[S]}+\frac{1}{V_{\max}} \qquad (1)$$

provided the concentration of I is known and the intercept on the horizontal axis (say Z) measured (see equation 2). Now for the IAA/inhibitor system to

$$K_I = -\frac{I}{(K_s/Z)-1} \tag{2}$$

be comparable with the 2:4-D/inhibitor system, the value of Z should be d instead of c (Fig. 4), that is Z should be smaller than its observed value. The affinity of IAA (i.e. $1/K_s$) should be b instead of a, that is $1/K_s$ should be smaller than its observed value. In equation (2), I is a constant; K_s should be larger and Z should be smaller than the observed values, therefore the denominator should be larger, and hence K_I should be smaller. So for the IAA/inhibitor system to be comparable with the 2:4-D/inhibitor system, the K_I of the former should be smaller than it actually is, or stated alternatively the K_I with IAA will be greater than the K_I with 2:4-D.

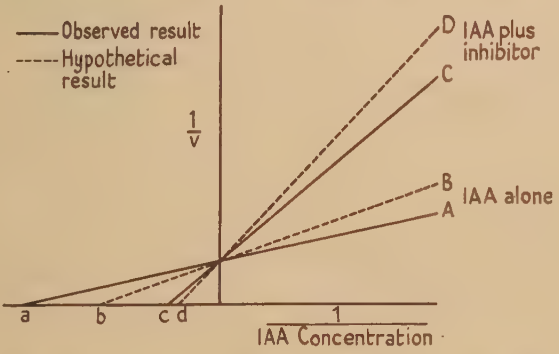

FIG. 4. Results of an hypothetical experiment with IAA and a competitive inhibitor. Relationship between the reciprocal of initial velocity of coleoptile section growth ($1/v$) and the reciprocal of IAA concentration is shown for IAA alone, and for IAA with a single concentration of inhibitor. See text for explanation.

The K_I values (molar) experimentally obtained by McRae and Bonner are shown below. It is seen that the K_I values with IAA are the greater, although with 2:4-DCA they are virtually equal. It should be pointed out that although McRae and Bonner consider the latter substance to be a competitive inhibitor, this opinion is not shared by Audus and Shipton (1952), nor by Thimann (reported by Wain, 1953), who consider it to be a growth inhibitor only. If this is true and 2:4-DCA is not an anti-auxin, but only a non-competitive inhibitor of cell elongation, then the K_I values of this inhibitor in the presence of IAA and 2:4-D should be independent of the relative intracellular concentrations of the three substances, and as such the two K_I values should be equal. This example is considered in detail in the next paragraph.

	4-CPIB	2:4-DCPIB	2:4-DCA
In presence of IAA	$3{\cdot}1\times10^{-6}$	$3{\cdot}8\times10^{-6}$	$1{\cdot}8\times10^{-5}$
In presence of 2:4-D	$2{\cdot}6\times10^{-6}$	$3{\cdot}2\times10^{-6}$	$1{\cdot}7\times10^{-5}$

The reciprocal form of the equation for non-competitive inhibition is shown below (equation 3: Dixon, 1953*b*). The intercepts on vertical and horizontal

$$\frac{1}{v} = \frac{1}{V_{\max}}\left(1+\frac{K_s}{S}\right)\left(1+\frac{\mathrm{I}}{K_\mathrm{I}}\right). \tag{3}$$

axes are $1/V_{\max}$ and $-1/K_s$ respectively (represented in Fig. 5 by graph *A*). When the inhibitor is present the intercept on the horizontal axis is still $-1/K_s$, but on the vertical it is $1/V_{\max}+\mathrm{I}/K_\mathrm{I}V_{\max}$ (Fig. 5, graph *C*). If one now applies the same approach as was used in the example of Fig. 4 and imagines the transport factor of IAA to be reduced until it equals that of 2:4-D, the hypothetical graphs *D* and *B* (Fig. 5) corresponding to *C* and *A* respectively will be obtained. Considering graphs *C* and *D*, the equations for these graphs (4) and (5) are shown below: $1/V_{\max}$ (*A* and *B* intercepts) is

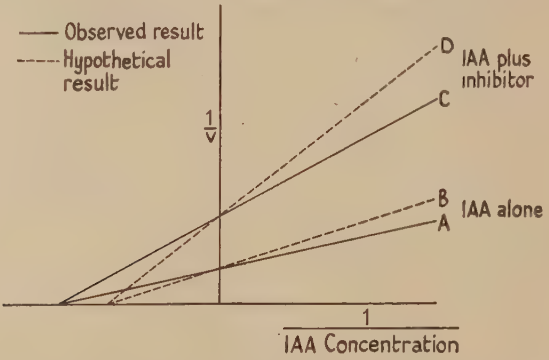

FIG. 5. Results of an hypothetical experiment with IAA and a non-competitive inhibitor. Relationship between the reciprocal of initial velocity of coleoptile section growth ($1/v$) and the reciprocal of IAA concentration is shown for IAA alone, and for IAA with a single concentration of inhibitor. See text for explanation.

$$\frac{1}{v^c} = \frac{1}{V_{\max}}\left(1+\frac{K_s^c}{S^c}\right)\left(1+\frac{\mathrm{I}}{K_\mathrm{I}^c}\right) \tag{4}$$

$$\frac{1}{v^d} = \frac{1}{V_{\max}}\left(1+\frac{K_s^d}{S^d}\right)\left(1+\frac{\mathrm{I}}{K_\mathrm{I}^d}\right) \tag{5}$$

identical in the equations for *C* and *D*, and the inhibitor concentration I is the same too. Also, at the position of the common intercept on the vertical axis of graphs *C* and *D*, $1/v^c = 1/v^d$ and $1/S^c = 1/S^d = 0$. So after equating (4) and (5) at this point and simplifying (equation 6), it is seen that K_I^c must be

$$1+\frac{\mathrm{I}}{K_\mathrm{I}^c} = 1+\frac{\mathrm{I}}{K_\mathrm{I}^d} \tag{6}$$

numerically equal to K_I^d. Therefore one may expect the K_I values of a non-competitive inhibitor, when determined in the presence of different hormones, to be equal.

McRae and Bonner consider their K_I values to be essentially equal in the presence of IAA and 2:4-D, thereby assigning the differences to experimental error. Although the assumptions made in the present paper to calculate the K_I values in the presence of IAA and 2:4-D are crude, the results of McRae and Bonner reflect expectation quite consistently, particularly if Audus's and Thimann's views on 2:4-DCA are correct. Furthermore, it has been stated earlier that the design of McRae and Bonner's experiments is poor to test the hypothesis advanced in this section, for the difference in transport between IAA and 2:4-D may not be large. A more critical test would be to calculate the K_I values of these three inhibitors in the presence of IAA and of IAN, or possibly 2:4-D and its corresponding nitrile. With these growth promoters one would expect the K_I values of the competitive inhibitors to show a considerable discrepancy from equality.

TABLE II

The observed free energy of formation of Michaelis complexes and the corresponding equilibrium constants are shown for Avena *coleoptile sections in solutions of three hormones. See text for further details*

Expt. No	Treatment	K_s(molar)	ΔG (cal./mole)
B244	IAN	$4{\cdot}2\times10^{-8}$	-10100
	IAN (sections apically blocked)	$6{\cdot}5\times10^{-8}$	-9850
	IAA	$1{\cdot}4\times10^{-7}$	-9400
B245	IAN	$9{\cdot}0\times10^{-8}$	-9650
	IAEt	$1{\cdot}3\times10^{-7}$	-9450
	IAA	$1{\cdot}7\times10^{-7}$	-9300

K_s and K_I values for a number of growth promoters and inhibitors have been measured by McRae and Bonner (loc. cit.). From these parameters they calculate the free energy of formation of *ES* and *EI* complexes, and thereupon proceed to make deductions on affinities of different parts of the substrate molecule for the receptor entity. From previous discussion in this paper it is clear that care must be taken in drawing conclusions from such data. To illustrate this point more concisely, values of K_s for the different treatments in Figs. 2 and 3 have been calculated (Table II), and from these, values of ΔG (free energy of formation of *ES*) have been deduced (Dixon, 1953*a*). In Expt. B244 the effect on K_s and ΔG of apically blocking the coleoptile sections with petroleum jelly is readily seen; and undoubtedly, had both ends of the sections been blocked instead of only the apical end, then the K_s and ΔG values would have been still closer to those for IAA. Thus, even with a compound which penetrates into the cells relatively easily, its ability to be transported through the coleoptile must still be borne in mind when interpreting data. In the following experiment (B245) it would seem at first that IAN has the greatest affinity for the receptor entity, followed by IAEt and then IAA. If the IAA hydrolysis theory is accepted for the mechanisms of action of IAN and IAEt, then these values of K_s and ΔG are little more than arbitrary measures of intracellular concentration of IAA: on the other hand, even if it is assumed that IAN and or IAEt are active *per se*, then with virtual certainty these parameters still reflect the permeability of the coleoptile tissues to these compounds to a considerable extent. Thus it is evident that before conclusions on substrate receptor entity affinities and bonding are drawn, it is first necessary to consider the relative abilities of the substrates to reach the active sites of growth within the cells.

INHIBITION OF CELL ELONGATION AT HIGH HORMONE CONCENTRATIONS

In previous studies (Bentley and Housley, 1953) it has been pointed out that growth inhibition, as explained by steric hindrance to two-point attachment between hormone and receptor entity, cannot account for the shape of the growth curve obtained with *Avena* coleoptile sections at high IAA concentrations. Subsequent to this publication, detailed American data became available (Foster *et al.*, 1952) and a more extensive reinvestigation of this work made possible.

These studies demand an exact knowledge of initial velocity of section growth, and of the shape of growth curves. Foster *et al.* (loc. cit.) obtained their initial velocities by section measurement after 12 hours, a linear relationship being observed over the first 18 hours' growth. This linear relationship was obtained over an IAA range of approximately 0·01–380 mg./l., the hormone being added to a basal medium of 3 per cent. sucrose and 0·0025 M. potassium maleate buffer (pH 4·5). These results, particularly at high IAA concentrations, contrast markedly with those of Bentley and Housley (loc. cit.), who show that *Avena* coleoptile sections are not only inhibited in a 100 mg. l. IAA solution, but that they have a maximum growth at approximately 6 hours, and after this time they start to shrink and lose their turgidity. To examine this phenomenon more fully, growth curves have been obtained for *Avena* coleoptile sections in solutions of IAA and IAN at high concentrations.

Concentrations of IAA and IAN used, times of section measurement, and results obtained are shown in Figs. 6 and 7. It is seen that at 300 mg./l. IAA and all higher concentrations of IAN and IAA, section response rises to a maximum and then declines. After maximum growth sections lose their turgidity, intercellular spaces in the coleoptile become filled with water, and the sections sink to the bottom of the dishes. In IAA the inner primary leaf changes colour from light green to yellow and in IAN to pink. It would appear that the permeability regulating mechanism of the coleoptile cells breaks down under the influence of high concentrations of hormone, thus permitting cell water to pass out into the external solution: this phenomenon results in cell and section shrinkage. Change in colour of the inner leaf suggests that the hormones at these concentrations are toxic to chloroplasts also.

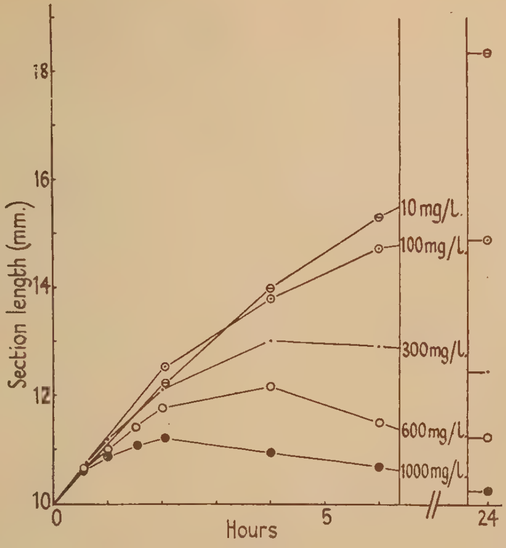

FIG. 6. Growth of *Avena* coleoptile sections in solutions of IAA over the first 6 hours, and final growth after 24 hours.

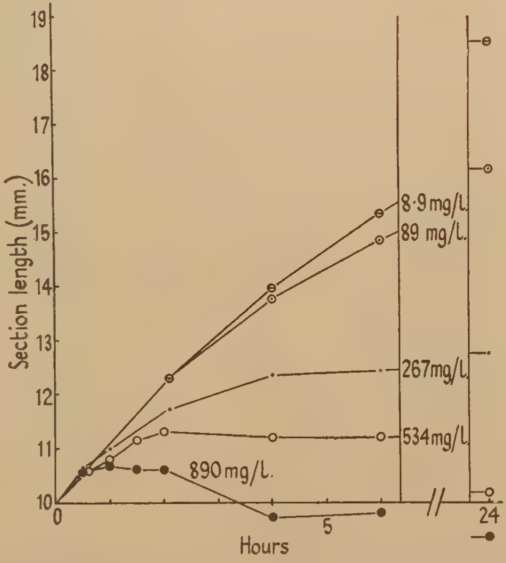

FIG. 7. Growth of *Avena* coleoptile sections in solutions of IAN over the first 6 hours, and final growth after 24 hours. Concentrations are molar equivalents of the IAA concentrations in Fig. 6.

DISCUSSION

The results clearly show that at the higher hormone concentrations, initial velocity of growth is only uniform for approximately the first 30 minutes: this period is too small for differences in rates of growth to be satisfactorily expressed; also an appreciable fraction of this period must be spent in the hormones reaching the active sites controlling cell elongation, thus growth over this period must be largely influenced by native auxin(s) of the coleoptile. It is obvious that initial velocities obtained under these conditions are of little value to the present problem: furthermore, they contrast markedly with the velocities claimed by Foster *et al.* (1952). The interpretation of the data of these workers in terms of the present type of growth curve is treated in detail by Bennet-Clark and Kefford (1954) and will not be considered here.

It would be expected that coleoptile sections inhibited at high auxin concentrations by steric hindrance to two-point attachment would be quite normal and healthy in appearance and would resemble sections in either distilled water or in very low concentrations of hormone. However, the present work has shown quite clearly that inhibition of final section length principally results from section shrinkage, the latter being a result of extreme toxicity. At increasingly lower hormone concentrations toxicity will also be present though to a progressively less extent: at these concentrations it is certain that the disappearance of external symptoms of toxicity is no criterion for assuming a normal healthy condition to exist, and that internal symptoms of toxicity will still occur and influence growth. Thus it is highly probable that at 100 mg./l. IAA and lower concentrations and at 267 mg./l. IAN and less, toxicity is still present. It is noteworthy that on another occasion 100 mg. l. IAA caused marked section shrinkage (shown in Fig. 1 of Bentley and Housley, 1953); also, it is of interest that von Guttenberg and Meinl (1952) report that 10^{-3} M. IAA (175 mg./l.) quickly destroys the protoplasm of the cells of *Rhoeo discolor*.

It would seem probable that steric hindrance to two-point attachment, if it occurs at all, only plays a minor role in causing growth inhibition, possibly at hormone concentrations which are only just supraoptimal to cell elongation.

THE USE OF FOOD FACTORS IN BASAL MEDIA

Important aspects of technique in kinetic treatment of *Avena* cell elongation arise from the work of Bonner and co-workers, and from Bennet-Clark and Kefford (1954). These workers used a basal medium of 3 per cent. sucrose, while Bonner also incorporated 0·0025 M. potassium maleate buffer (pH 4·5) into test solutions.

McRae, Foster, and Bonner (1953) cite the 18-hour linear portion of their growth curves as evidence that the total concentration of receptive sites in the cell remains constant with time. In contrast with this, coleoptile section curves of Bentley and Housley (1953) obtained with IAN and IAA alone clearly show that a linear relationship only occurs for the first 2 hours at the most, and that after 5 or 6 hours one is no longer even justified in assuming that the curve is approximately linear. However, the addition of sucrose to hormone solutions under the conditions in this laboratory has the effect of increasing the initial period over which a linear response is shown (unpublished results). Thus it would seem that the conclusions of McRae *et al.* (loc. cit.) have been markedly influenced as a result of sucrose being used as a basal medium.

Bennet-Clark and Kefford (loc. cit.), by the addition of 3 per cent. sucrose, obtained sigmoid growth curves: they consider that the American workers failed to obtain similar curves through the presence of potassium ions in the buffer. However, these sigmoid curves proved some embarrassment to them in expressing the initial velocity of section growth.

The function of sucrose in enhancing cell elongation is not understood, although in the past it has been referred to as a food factor (see Bentley, 1950). It would therefore appear that the use of sucrose is an unnecessary complicating factor and is not to be recommended.

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Factors controlling Flowering in the Chrysanthemum

IV. THE SITE OF VERNALIZATION AND TRANSLOCATION OF THE STIMULUS

W. W. SCHWABE

Research Institute of Plant Physiology, Imperial College of Science and Technology, London

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SUMMARY

By confining low-temperature treatment to the growing tip of the Chrysanthemum plant, the apex has been shown to be the seat of perception of vernalization, a result which is in full accord with earlier experiments on other plants requiring vernalization. Experiments on the translocation of the stimulus through mature tissues involving stock/scion and 'approach' grafts with or without defoliation gave negative results. By decapitating in turn the main axis and the resulting laterals produced, it has been shown that the stimulus is passed on from existing apices to laterals of up to the seventh order at least, lateral apices which were formed well after the time of chilling. These results are discussed in relation to prior work on the existence of a specific vernalization substance as well as a flowering hormone and their translocation.

INTRODUCTION

VERY soon after the discovery of the vernalization requirement of certain biennial plants had been made, investigations were begun with the object of locating the seat of action of the cold treatment. While the leaves were shown to be the organs perceiving the daylength stimulus, it appeared from these experiments that the cold treatment must be given to the growing-point itself. By circulating cold water round the base of the crown of plants, Curtis and Chang (1930) and Chroboczek (1934) showed that in celery and beet respectively it is fully sufficient to cool this part of the plant to secure the vernalizing effect, while cooling of the lower parts of the root was ineffective. Gregory and Purvis (1938) have shown that the rye embryo alone needed to receive cold treatment and that even parts of the embryo were susceptible to treatment (Purvis, 1940), so long as the growing-point was included. In these studies, the whole of the tissue giving rise to the final plant actually received the cold treatment. Melchers (1936) has also shown that the tissue of the biennial variety of *Hyoscyamus niger*, which needs to be chilled in order to induce flowering in the first season, is situated in the terminal apex and not in the root stock. His evidence was obtained in experiments in which growing-points of vernalized and unvernalized plants were exchanged by grafting, and these

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indicated that neither the leaves nor the roots but only the growing-points are capable of perceiving the stimulus of cold treatment.

In order to repeat these experiments and to define the seat of stimulation by vernalization for the Chrysanthemum also, some of the experiments to be described were set up. The results thus obtained raised the further question of the translocation of the stimulus in the plant. That an actual flowering stimulus, often identified with a specific flowering hormone, can be translocated about the plant appears to be general and has been shown in many experiments (cf. the recent reviews by Lang, 1952, and Leopold, 1951). The position as regards the vernalization stimulus is considerably less clear. In the winter cereals and such biennials as *Hyoscyamus* and beet vernalization must normally be followed by long-day treatment and to some extent it is possible to distinguish between the after-effects of daylength and temperature.

On the basis of grafting experiments, Melchers (1938, 1939) has concluded that the vernalization effect can be regarded as resulting in the production of a substance, tentatively called 'vernalin', which is also transmissible within the plant and, by means of grafts, from one plant to another. Wycherley (1952) believes that the hypothetical vernalization product is immobile. Gardner and Loomis (1953) also failed to obtain translocation in *Dactylis glomerata*. Purvis and Gregory (1937) have postulated autocatalytic multiplication of the hypothetical substance, for in vernalized winter rye the numerous tillers arising after termination of the cold treatment are also vernalized. This interpretation was also supported by data on the 'intensity' of vernalization after different periods of cold treatment (Purvis, 1948).

Thus, in addition to those experiments set up to locate the seat of vernalization in the Chrysanthemum, a number of grafting experiments were also undertaken to attempt some clarification of the problem of transmission for the Chrysanthemum.

METHODS AND RESULTS

The general methods of growing and raising the plant material[1] were those described in the first part of this series. The daylength given throughout the experiments was 8 hours' exposure to full daylight. The vernalization treatment of entire young plants was given in a refrigerator kept at an average temperature of approximately 5° C.; the method of apical vernalization is described below. Effects on flowering behaviour were again estimated from leaf-number data and the time required for the production of the macroscopically visible inflorescence bud.

Apical vernalization. In the first few experiments an attempt was made to confine the cooling treatment to the apical growing-point while keeping the rest of the plant at ordinary greenhouse temperatures. Without the use of elaborate apparatus this was achieved by the following method. The young unvernalized cuttings were grown to a somewhat larger size than has been the normal practice in this work. They were then placed in a warm frame with the apical buds exposed through narrow apertures cut in the glass pane above the plants. The tips of the plants were then sealed in by means of plasticine. This necessitated the removal of some leaves below the growing-point and the remaining leaves had to be held away from the glass surface by wire loops to prevent them from becoming cooled by contact. The control plants were placed in a similar position but with their apices just below the glass surface, and some of their leaves were likewise removed. In order to make use of the low outdoor temperatures, these experiments had to be carried out during the winter months; in view of the proximity of all plants to the glass it was not possible to prevent some lowering of the temperature near the glass itself, and in the first of these experiments a marked degree of vernalization resulted in the controls also (cf. the mean minimum temperatures shown in Table I). The results of the two experiments shown in Table I indicate clearly, however, that apical vernalization alone is sufficient to cause greatly accelerated flowering and a large reduction in leaf numbers.

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[1] The Chrysanthemum variety used in the majority of the experiments described in this and the preceding papers of this series was taken over by the author from stocks kept at the Institute labelled 'Indian Summer'. During 1953, however, an attempt has been made to check this identification and it would appear that the correct name should have been 'Sunbeam' and not 'Indian Summer'. Of course, this does not affect any of the results presented.

TABLE I

Effect of vernalizing apical bud. Varieties 'Sunbeam' and 'Cossack'. Growing-point chilled for 4 weeks

Variety	Treatment	Number of replicates	Number budded	Leaf no. increment to budding	Days to budding	Mean minimum temperature at bud level.
'Sunbeam'	Control	7	7	32·4	136·5	9·1° C.
	Gr. pt. chilled	8	8	21·6	112·1	2·3° C.
	t (0·001 = 4·22)			6·6	14·2	
'Cossack'	Control	8	5	79·0 (86·0)*	143·0	14·5° C.
	Gr. pt. chilled	8	8	40·5	81·0	3·8° C.
	t (0·001 = 4·44)			10·4	19·9	

* Leaf no. of vegetative plants.

Although these experiments confirmed the suggestion that apical vernalization alone is sufficient to establish the effect, they did not exclude the possibility that vernalization of parts of the plant basal to the apex might be equally effective. In order to test this, a similar experiment was set up in which the the growing-points of the plants (approx. 1 in.) were admitted into the warm greenhouse while the major part of the stem and the roots were kept out of doors. As controls plants were used whose apices were cooled as in the previously described experiments, as well as replicates kept wholly inside or outside the greenhouse. The details of treatment were similar to those described except that the perforated glass window in this case was a vertical pane, which proved quite satisfactory in view of the diageotropic growth habit of unvernalized plants. A preliminary experiment with rather variable plant material was used as a trial run, to be followed by a second with more uniform plant material. The results of both experiments are shown in Table II, but significant differences have been calculated for the second only.

The flowering behaviour of the plants whose apices were kept warm while the rest of the plant was chilled shows clearly that the effect of chilling parts other than the apex is of very little significance compared with that obtained by chilling the main growing point. In fact, it is practically certain that the eventual flowering of three replicates of this treatment was due to the temperatures just inside the glass being slightly lower, rather than due to any transfer of stimulus from the base of the plant. In two of these three plants lateral shoots developed on the chilled part of the stem, and these formed inflorescences at about the same time as the apically vernalized treatments and 5 and 12 weeks respectively before the main axes on these plants budded. The leaf-number increments and times required for bud formation are very similar as between those pairs of treatments in which the growing-points were subjected to similar temperatures.

During the actual treatment, all those growing-points kept at the higher temperature continued to expand new leaves, while those at the lower temperature—as might be expected—were much retarded. This retardation also affected their apical dominance, numerous lateral shoots developing on the part of the stem kept in the greenhouse. Such laterals were subsequently removed to prevent their exerting dominance effects on the terminal growing point.

TABLE II

Effect of chilling the growing-point or mature part of plant. Variety 'Sunbeam'. Preliminary experiment 4 weeks' vernalization, repeat experiment 6 weeks'

Preliminary experiment:	Whole plant in greenhouse	Growing point in greenhouse	Whole plant chilled	Growing point chilled	Sig. diff.
No. of replicates	5	5	5	4	—
No. of plants budded . . .	1	0	4	3	—
Leaf number increment to budding*	45 (35·2)	(41·8)	29·3 (31)	31·0 (30)	—
Days to budding from beginning of expt.	118	—	131·0	112·3	—
No. of leaves produced during treatment	10.6	9.0	2·0	2·0	—
Mean minimum temp. during treatment	16·1° C.		2·4° C.		
Repeat experiment:					
No. of replicates	5	5	5	5	—
No. of plants budded . . .	0	3	5	5	—
Leaf number increment to budding*	(72·4)	56·2 (62·0)	30·0	35·2	9·2
Days to budding from beginning of expt.	—	154·0	91·6	100·6	20·4
No. of leaves produced during treatment	15·8	12·8	6·6	7·8	—
Mean minimum temp. during treatment	16·8° C.		3·6° C.		

* Increment for vegetative plants in parentheses.

Translocation of the vernalization stimulus through mature parts of the plant. While producing further evidence for the apical localization of the tissue sensitive to vernalization, these experiments also seemed to show that little or no translocation of the stimulus occurs from the basal to the more apical regions of the plant.

In order to investigate this aspect more directly, a number of grafting experiments were undertaken. In these grafts young vernalized plants were used as donors—but well before they had themselves budded. This was done in order to differentiate, if possible, between the effects of translocation of the vernalization stimulus and one which might result after flower formation by the donor.

In the first experiments, the grafts were made by cutting both scion and stock obliquely across the stem and binding them together with string and a short length of bandage, which was then covered by grafting wax. Later thin rubber bands (as used in golf balls) were used to tie the graft partners; also wedge-shaped scions placed into V-cuts in the stock were found to be somewhat more convenient. Each scion had one or two leaves cut across the tip, which was then immersed in a small vial containing water in order to prevent wilting. The whole plant was finally covered by a small bell jar for approximately 14 days until the grafts had taken. After this period, the plants were hardened off and the graft junctions freed from covering material.

The first of these experiments was designed to confirm the observation described above that the apical part of the plant appeared uninfluenced by the state of vernalization of the base. The experiment consisted of reciprocal grafts between the following pairs: vernalized scion on unvernalized stock (*V*/*H*), unvernalized scion on vernalized stock (*H*/*V*), vernalized scion on vernalized stock (*V*/*V*); and unvernalized scion on unvernalized stock (*H*/*H*). The latter two types of graft served as controls to show that the actual procedure of grafting did not affect the state of vernalization of the scion. The results of this stock/scion experiment are presented in Table III, and from this it will be obvious that the flowering behaviour of the scions has remained unaffected by the state of vernalization of the stock. Vernalized scions budded and unvernalized scions remained vegetative. Leaf number data and the average internode lengths of the scions indicate the same result.

In the two reciprocal sets of grafts one or two laterals were allowed to develop from the stock part of the composite plant in order to discover whether transfer of the stimulus had perhaps gone on in a basipetal direction. But again no evidence for such transfer was found. All laterals on the unvernalized stocks remained vegetative. Two vernalized stocks, however, did produce short flowering laterals, while five others again remained vegetative. The failure of these laterals to flower is attributed to the de-vernalization of lateral buds which will be discussed in a future paper of this series.

In a further grafting experiment young vernalized and unvernalized plants were rooted side by side and then 'approach-grafted'. The graft pairs were: vernalized with vernalized (*V*-*V*), unvernalized with unvernalized (*H*-*H*),

TABLE III

Translocation of the vernalization stimulus from stock to scion. Variety 'Sunbeam'

	H/H	*V/V*	*V/H*	*H/V*	Sig. Diff.
No. of successful grafts	9	8	7	7	—
No. of scions budded	0	7	7	0	—
Leaf number increment to budding on scion*	(37·8)	23·7 (43)	22·3	(43·9)	5·5
Days to budding from grafting	—	51·3	51·3	—	8·4
Mean internode length of scion (cm.)*	(0·26)	0·93 (0·29)	0·81	(0·27)	0·15
No. of plants with vegetative laterals on stock	—	—	7	5	—
No. of plants with budded laterals on stock	—	—	0	2	—

* Leaf numbers and internode lengths for vegetative plants in parenthesis.

and vernalized with unvernalized (*V-H*). In treatments *H-H* and *V-V*, in which both partners had the same pre-treatment, one partner, arbitrarily called the 'receptor', was defoliated. In treatment *V-H*—where the vernalized partner was expected to bud normally—half the unvernalized partners were defoliated at the time of grafting in order to facilitate any transfer of the stimulus. However, as there was no difference in flowering behaviour between these two treatments, the data have been bulked for both sets in Table IV, and here the 'receptors' are the unvernalized partners.

TABLE IV

Effect of 'approach-grafting' vernalized and unvernalized plants. Variety 'Sunbeam'

	H-H		*V-V*		*V-H*		Standard deviation
	Donor	Receptor	Donor	Receptor	Donor	Receptor	(per plant)
No. of replicates	5	4*	5	5	10	10	—
No. of plants budded	0	0	5	1	10	0	—
Leaf number to budding†	(42·8)	(28·2)	24·4	21(30·5)	24·4	(33·8)	±4·2
Days to budding from grafting	—	—	43·4	89	42·9	—	±1·6
Mean internode length at time of budding (cm.) (in vegetative plants at end of experiment)	— 0·30	— 0·22	1·03 —	0·55 0·25	1·05 —	— 0·22	±0·09

* One plant died. † Leaf numbers for vegetative plants in parenthesis.

The results of this 'approach-graft' experiment again indicate no transfer of the vernalization stimulus, and none of the unvernalized partners had formed inflorescence buds by the end of the experiment. There was clearly no need to establish statistically significant differences, but as a measure of the variability of the material standard deviations are shown in Table IV. However, it is also clear from the data for the grafts between two vernalized partners (*V-V*) that where one partner was defoliated and thus retarded in its own growth it was gradually overshadowed by the vigorously growing donor and thus became devernalized. Only one of the receptors of this type of graft finally budded and only after a much longer time than the non-defoliated partner. Thus it would appear possible that the effect of any 'approach-graft' experiment was bound to be stultified through devernalization supervening, and it might be argued that even if any translocation of the stimulus had occurred as a consequence of defoliation it would have been annulled again subsequently.

A further attempt was therefore made to obtain transfer of the vernalization effect in a stock/scion graft experiment in which half the scions were defoliated as soon as the graft had taken, in order to facilitate translocation from the stock; in the control set of plants, all leaves were left on the scions. Vernalized scions grafted on to unvernalized stocks were similarly treated and served as further controls. All the plants used in this experiment had received a very slight degree of vernalization prior to treatment, but this was not regarded as a disadvantage since the comparison to be made was merely between the effects of defoliation and its absence. The experiment was carried

TABLE V

Effect of defoliation of the scion on translocation of the vernalization stimulus from stock to scion. Variety 'Sunbeam'

	V/H control	*V/H* defol.	*H/V* control	*H/V* defol.	*H*—not grafted	Sig. diff.
Series A:						
No. of successful grafts .	7	8	6	7	—	—
No. of scions budded .	7	8	6	7	—	—
Leaf number to budding on scion . . .	22·0	24·6	38·1	40·4	—	4·5
Days to budding from grafting . . .	59·4	66·2	87·2	101·9	—	10·1
No. of budded laterals on stock . . .	1	0	2	2	—	—
Series B:						
No. of successful grafts .	10	10	9	10	10	—
No. of scions budded .	10	10	6	6	9	—
Leaf number to budding on scion* . . .	23·3	22·7	41·1 (45·0)	39·1 (43·5)	35·1 (56)	8·0
Days to budding from defoliation (i.e. 15 days from grafting) . .	37·1	39·9	71·0	78·5	45·9	21·1
No. of stocks with lateral shoots . . .	10	10	4	5	—	—
No. of stock laterals budded . . .	5	4	4	5	—	—
Leaf number to budding on stock laterals . .	32·0	27·5	20·2	21·8	—	—

* Leaf numbers for vegetative plants in parenthesis.

out in two series, in the second of which another set of ungrafted controls was included to determine the degree of partial vernalization. The results shown in Table V give no indication whatever that defoliation of the scions immediately after the graft union had become established caused any acceleration of flowering (and hence transmission of the vernalization stimulus) compared with the undefoliated controls. The ungrafted controls of series B also show that such flowering as occurred in the *H*-scion treatments was due to the slight vernalization before grafting. A few lateral shoots started at the time of grafting were allowed to grow on from the basal parts of the plants. In series B, all these early laterals produced on vernalized stocks budded, while those on the *H*-stocks showed again only the same slight degree of vernalization as the *H*-scions and the ungrafted controls. Their leaf numbers also were much higher than those of the *V*-stock laterals.

Transmission of the vernalization stimulus through meristematic tissue. Purvis (1948 and unpublished) has carried out some experiments in which the main shoots of vernalized rye plants were removed and the tillers arising were again cut off; none the less, the plants produced further tillers flowering normally, a result which demonstrated that even tillers arising from axillary buds non-existent at the time of vernalization behaved as if they had been subjected to such treatment.

In order to test the applicability of these results to the Chrysanthemum, two experiments were carried out involving one or more successive decapitations of fully vernalized plants. In the first of these each decapitation was carried out after production of five new leaves, a single lateral being allowed to grow on after each such treatment. Originally this experiment was designed to compare the effects of 1, 2, 3, 5, and 7 such decapitations. After the last of these, the uppermost lateral was to be allowed to complete its growth. However, during the experiment it became clear that before the third decapitation could be carried out a number of plants budded, and in consequence one set of plants was decapitated after the second-order lateral had budded, while another set was so treated after producing only three leaves on the second-order lateral. All the plants in this experiment budded regardless of treatment; the mean numbers of leaves produced before bud formation are shown in Table VI.

TABLE VI

Effect of successive decapitations on the state of vernalization of lateral apices. Variety 'Sunbeam'. Eight replicates

	Number of decapitations:			
	1	2	3 (after budding)	3 (before) budding)
Leaf number to budding on final lateral branch	16·3	9·0	4·4	7·8
Leaf number on rest of plant .	5	10	15	13
Total	21·3	19·0	19·4	20·8

In the second experiment, all conditions were similar except that the decapitations were carried out after every second leaf and in this way it was possible to extend the number of treatments to the production of branches of the seventh order. Beyond this, budding again occurred in many plants before two leaves had separated sufficiently from the terminal bud to permit a further decapitation. The results derived from only two replicates per treatment are shown in Table VII.

TABLE VII

Effect of successive decapitations on the state of vernalization of lateral apices. Decapitation after production of two leaves per shoot. Variety 'Sunbeam'. Two replicates

	Number of decapitations							
	1	2	3	4	5	6	7	Sig. diff.
Leaf number to budding on final lateral branch . .	25	21	15	10	8	3	6	
Leaf number on rest of plant	2	4	6	8	10	12	14	
Total	27	25	21	18	18	15	20	7·4

The results of both these experiments fully confirm the similar experiments with rye in showing that the vernalization stimulus is passed on to lateral apices arising after the treatment of the original meristems has ended; and the effect is not lost and is apparent in branches of the seventh order produced after the end of cold treatment. The fairly constant total number of leaves produced regardless of the degree of branching before formation of the inflorescence bud is also worthy of note.

DISCUSSION

As regards the locus of perception of the vernalization stimulus, the results described above fully confirm those of Chroboczek (1934) and Melchers (1936), obtained with long-day biennials, and those of Purvis and Gregory (1938) with winter rye. In the Chrysanthemum also, it is the apical meristem which requires to be vernalized and which reacts to the stimulus. This result raises the further question whether the effect of vernalization remains localized at the original site or whether it is translocated about the plant.

There is overwhelming evidence that the daylength stimulus is transmissible (cf. Lang, 1952), but very few experiments indeed have been carried out on the possibility of transport of the vernalization stimulus. Melchers (1939) has claimed that the fact that whole tops or single leaves of 'Maryland Mammoth' tobacco grafted on to biennial *Hyoscyamus* plants cause the latter to flower under daylength conditions in which this 'donor' plant is itself incapable of flowering, constitutes evidence of the presence and translocation of a vernalizing substance, termed 'vernalin', always present in the annual tobacco. Such translocation is the only evidence we possess for the material character of both the hypothetical flowering and vernalizing substances. The present experiments with the Chrysanthemum, however, do not suggest that translocation of the vernalization stimulus takes place through the mature tissues of this plant. Of course, these results suffer from the disadvantage of all negative results; and it may well be that more positive evidence for the substantial character of the vernalizing effect will yet be found. On the other hand, there is no doubt that new axillary meristems arising after the end of the cold treatment behave as if fully vernalized. Gregory and Purvis (1937) also believe that vernalization results in the formation of a substance, and their observation of the transmission of the stimulus to lateral apices of a higher order than were present at the time of cold treatment is seen to be fully confirmed in the present decapitation experiments. However, in the present experiments, as in theirs, these higher-order lateral apices were actually derived from tissue which had itself been vernalized; and though this may well constitute evidence of an increase in the number of vernalized cells and total mass of vernalized meristematic tissue, it does not necessarily prove the substantial character of the stimulus. It is to be hoped that further work may supply more stringent evidence. Quite recently Purvis and Gregory (1953) have put forward evidence for an acceleration of the development of unvernalized rye embryos by means of extracts made from several times their number of vernalized embryos. The degree of acceleration so far has only been small, up to the level of whole unvernalized grain, but if this effect can be increased it would go a long way towards proving that a specific substance is formed as the result of cold treatment.

In this connexion, reference may perhaps be made to some claims still cited frequently in the literature that, in the Chrysanthemum, translocation of the daylength stimulus has been demonstrated in the absence of any living tissue union such as has been shown necessary in, for example, *Xanthium* (Withrow and Withrow, 1943).

The first of these reports was by Moshkov (1937), and since then Gerhard (1940) has claimed to have repeated these experiments with success. His results, however, are capable of a different interpretation which avoids the need for postulating translocation of the flowering effect.

Briefly, his main treatment consisted in removing the blades from the uppermost few leaves of Chrysanthemum plants kept in long day and placing the cut petioles in intimate contact with leaves cut from plants grown in short day. These 'donor' leaves were renewed regularly, while all new leaves produced on the treated plants themselves, and also lateral shoots, were removed continuously. The controls consisted of several defoliation treatments designed to be generally similar to the main treatment but without apposition of leaves. Eight of the twelve replicates of the experimental series had produced potential inflorescence buds several weeks before the start of the experiment but these failed to develop into open inflorescences in the long-day conditions given. These eight plants later failed to 'respond' to the short-day leaf treatment The remaining four replicates produced buds some time after treatment. The author denies that these four plants had initiated these buds before treatmen and—*post hoc, ergo propter hoc*—claims that their subsequent behaviour was therefore due to transmission of the daylength effect from the short-day leaf blades adpressed to the petioles. The control plants behaved similarly to the eight experimental replicates which had budded prior to treatment and did not initiate new inflorescences. As a confirmatory experiment, the author treated plants which had produced such premature inflorescence buds with short day and found that they required a much longer time before initiating inflorescence buds on their leading laterals than other plants which had not previously budded on their main shoots. From this, one would infer that non-budded plants are regarded by Gerhard as more sensitive to the short-day stimulus. The alternative explanation of these results is based on the fact that Chrysanthemum plants *will* eventually produce potential inflorescence buds in long-day or even continuous light, but these buds fail to develop if the long-day treatment is continued, unless all lateral shoots are removed continuously (Schwabe, 1950 and 1951). Thus the crucial four plants in Gerhard's experiment were presumably just late in budding and the increased development of the potential inflorescence buds noted by the author would be a consequence of the continuous removal of all lateral shoots—a treatment by which even open inflorescences can be produced in long day. The delay between the time of producing an inflorescence bud on the main shoot and the development of another crop of such buds on the next set of axillaries as seen on the other eight replicates is a normal feature of the growth of the Chrysanthemum plant in any daylength and in part forms the basis of the 'stopping' treatment used by the grower. Thus Gerhard's results would not appear to be good evidence for a transmission of the flowering stimulus without tissue union.

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An Electron Microscope Study of Cysts in the Saprolegniaceae

HANS MEIER[1] AND JOHN WEBSTER
Botany Department, The University of Sheffield

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WITH 4 PLATES

SUMMARY

The structure of the zoospore cysts of various members of the Saprolegniaceae has been studied by electron microscopy. In *Saprolegnia ferax*, *S. dioica*, in *Isoachlya eccentrica* and *I. unispora* the primary cysts were smooth, whilst the secondary cysts bore stalked double-headed hooks. In *S. parasitica* the secondary cysts bore tufts of longer hooks. In *Protoachlya*, *Achlya*, and *Brevilegnia* neither type of cyst bore hooks. In *Dictyuchus sterile* the secondary cysts bore large spiny projections. The primary cysts in many species bore tufts of radiating hairs, and it is suggested that these are the remains of ciliated flagella.

INTRODUCTION

MANTON, Clarke, and Greenwood (1951) showed that the zoospore cysts of *Saprolegnia ferax* (Gruith) Thuret bear 'delicate double-headed hooks on long slender stalks'. It was assumed without detailed study that the membranes in question were from primary cysts. We have studied a wider range of material from the Saprolegniaceae in order to discover whether such structures were present in all its members, and whether hooks occurred on both first- and second-stage cysts. The study is incomplete, but as there is no immediate prospect of continuing it, the results obtained are presented here since they seem to be sufficiently interesting to stimulate further investigations elsewhere.

Most of the cultures used were obtained from the Centraalbureau voor Schimmelcultures, Baarn, but isolates were also obtained from twigs and animal substrata in streams and ponds near Sheffield. When free from bacteria, the fungi were grown on plates of maize extract agar. Sterilized (autoclaved) hemp seeds were then placed on the surface of the culture, and after the fungus had made vigorous growth on the hemp seed, the seed was transferred (one seed per dish) to small Petri dishes containing sterile distilled water, which was usually then incubated at 23° C. Ripe sporangia were formed within a few hours or, sometimes, 2 to 3 days. Once these were obtained the procedure varied from species to species and is described below.

The usual techniques were used in the electron microscopy. Collodion was used as a supporting film, and the specimens were shadowed with gold-palladium at an angle of about 20°. The living material was usually mounted directly on the grid, dried, and shadowed. When possible, observations were

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[1] Now at Institut für allgemeine Botanik, Eidgenössische Technische Hochschule, Zürich

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made on empty cyst membranes, which gave better results than full cysts. After preliminary trials no attempts were made to fix the specimens. The instrument used was the Metropolitan Vickers E.M. 3 housed in the Metallurgy Department of the University of Sheffield. All the electron micrographs were taken at 75 kV. The magnifications given are approximate.

A. THE DIPLANETIC GENERA OF THE SAPROLEGNIACEAE

These forms include fungi in which there are two morphologically distinct swimming-stages. The primary zoospore is pyriform and apically biflagellate. After a motile phase the spore encysts. We have used the term 'primary cyst' for the cyst formed at the end of the first swimming-stage. The primary cyst germinates to produce a secondary zoospore, reniform and laterally biflagellate. After swimming for some time the secondary zoospore encysts forming a secondary cyst, which may germinate by means of a germ-tube or may form a further secondary-type zoospore (repeated zoospore emergence). Since many of the so-called diplanetic forms are in reality 'polyplanetic' due to repeated emergence, Höhnk (1933) proposed the term 'dimorphic' for these forms, thus placing emphasis on the different morphology of the two types of zoospore. These genera include three well-distinguished groups:

1. *Saprolegnia*, *Isoachlya*, *Pythiopsis*,[1] and *Leptolegnia*, whose ovoid or pip-shaped primary zoospores escape separately from the sporangia into the water and swim away.
2. *Achlya*, *Aphanomyces*, and *Plectospira*, where the first swimming period is confined to the sporangium and the spores encyst at its mouth.
3. *Protoachlya*, where some of the spores escape as in the first group, whilst the remainder encyst at the mouth of the sporangium as in the second group.

Saprolegnia

The species examined were *S. ferax*, *S. dioica* de Bary, and *S. parasitica* Coker. *S. ferax* was studied from three different sources: an isolate used by Manton *et al.* (1951) in their own electron microscope studies, and isolations by Ivimey Cook and by Moreau supplied by C.B.S. All these isolates and also *S. dioica* (from C.B.S.) showed the same cyst structures. When empty cyst membranes were picked up with a fine pipette from a 2–3-day-old water culture and examined, two different kinds of cyst were always found (Fig. 1). Some had the stalked double-headed hooks previously described by Manton *et al.* (1951) and some were devoid of hooks but often bore a tuft of radiately arranged simple hairs (Figs. 1 and 2). These latter cysts sometimes bore very fine hairs scattered over the whole surface (Fig. 2). Fine hairs were also sometimes found on cysts with hooks (Fig. 3). It is possible that these hairs are merely thicker cellulose fibrils of the cyst wall.

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[1] Coker (1923) and Fitzpatrick (1930) consider *Pythiopsis* to be monoplanetic, but following Harvey (1925) and Höhnk (1933) we regard the genus as diplanetic. Since it is possible to find occasional empty primary cysts we infer the presence of a second motile stage.

The fact that two different types of cyst could be detected led us to suspect that the primary and secondary *cysts* might be dimorphic in addition to the swimming spores. Therefore we sought a technique which would separate primary and secondary cysts. A single almost ripe sporangium from a water culture was separated from the mycelium and placed in a hanging drop. The spores were usually discharged soon afterwards, especially if aerated water was used as recommended by Cotner (1930). The primary zoospores swam for periods varying from a few minutes to an hour, rotating in a counter-clockwise direction as described by Salvin (1941), then stopping suddenly and encysting. A few primary cysts formed secondary zoospores, many germinated by means of a germ-tube, and some did not germinate at all. Thus it was rather difficult to transfer a reasonable number of empty cysts to a grid. However, photographs were taken of full primary cysts, but hooks were never found on them, although the tufts of radiately arranged hairs described above were frequently seen. A more suitable method of obtaining empty primary cysts was to remove the single hemp seed with its mycelium from a water culture shortly after the first sporangia had discharged their spores, and before empty membranes were observed. In this way we ensured that only primary zoospores or full primary cysts remained in the dish. Most of the cysts lay at the bottom of the dish. The upper layers of water were pipetted away and replaced with freshly aerated sterile distilled water. A few hours later a number of empty primary cysts were found and examined with the electron microscope. *Hooks were never found on them.*

To separate secondary from primary cysts the method described by Manton *et al.* (1951) was adopted. From a water culture the mycelium was removed a few hours after zoospore discharge had begun. The suspension of swimming and encysted spores was transferred to a sterile test-tube 15 cm. long and 1·5 cm. in diameter. The tube was incubated overnight at 23° C. A dense suspension of secondary zoospores was found in the uppermost centimetre of water. Samples of this upper layer were transferred to a hanging drop in order to observe encystment. All cysts observed in such preparations bore hooks.

We therefore conclude that in *S. ferax* and in *S. dioica* the primary cysts do not bear hooks, although they may have radiating tufts of hairs. Only the secondary cysts bear hooks. These conclusions amplify and correct those of Manton *et al.*, who have also sent us a private communication to the effect that their 'original preparation did not explicitly exclude the presence of secondary cysts, which could therefore have been present.'

Saprolegnia parasitica

The species was twice isolated by us: from a stream and from a dead roach in a pond near Sheffield. It was also obtained from C.B.S. All three isolates showed the same peculiar cyst structure. The technique described above for separating the two types of cyst was used with success in *S. parasitica*.

In hanging drops containing a single sporangium some of the primary zoospores encysted within 2 minutes of discharge. Most of the spores, however, swam in small circles in a counter-clockwise direction for 15–60 minutes. Then, swimming quickly in ever decreasing circles either clockwise or counter-clockwise, they would stop suddenly and encyst. The primary cysts bear simple hairs as in *S. ferax* and *S. dioica*, but they are much longer (see Fig. 4). Sometimes also a radiately arranged tuft of hairs can be found as shown in Fig. 5, where the tuft is slightly detached from the cyst membrane. The primary cysts may germinate directly by a germ-tube (expecially in hanging drops) or they may give rise to secondary zoospores. These leave the cyst within 2–3 seconds, then remain stationary just outside the empty membrane. Twenty to thirty minutes later they begin to move convulsively, become more reniform, and suddenly swim away, moving directly rather than in circles. Before encystment they gradually swim more slowly until they finally come to rest. Such a spore in the phase of encystment is shown in Fig. 6. Here one flagellum, the ciliated flagellum, is still attached, and a firm wall has probably not yet been formed. In this specimen, the formation of *tufts* of long thin hairs bearing hooks at their tips is also taking place. Although these hairs have probably not yet reached their final length (compare Figs. 6 and 10), the hooks are already visible. The tufts seem to arise (Fig. 6) on small elevations, but these may be artefacts caused by uneven shrinking of the spore during preparation. The number of hairs in each tuft varies from 4 to 13. Fig. 10 shows a full secondary cyst, whilst we have preparations of empty secondary cysts, indicating that repeated zoospore emergence has occurred. Almost all the stalks of the hooks are bent backwards, and occasionally the hooks appear to be attached to their own cyst wall. It is possible that the recurvature is also an artefact.

Isoachlya

I. eccentrica Coker, *I. unispora* Coker & Couch, and *I. monilifera* (de Bary) Kauffman, obtained from C.B.S., were examined, but detailed studies were only possible in the first two species, due to difficulties in obtaining secondary zoospores in *I. monilifera*. *I. eccentrica* and *I. unispora* closely resembled each other in cyst structure and also *S. ferax* and *S. dioica*. The cysts were dimorphic, the primary ones bearing small simple hairs and sometimes radiate tufts of hairs (Fig. 8). The secondary cysts bore hooks of the *S. ferax* type (Fig. 9).

Pythiopsis

P. cymosa de Bary and *P. intermedia* Coker & Harvey from C.B.S. were studied. In *P. cymosa* it is claimed that the only swimming stage is the primary zoospore, which germinates by a germ-tube after encystment. In *P. intermedia* Höhnk (1933) claims that the primary cysts give rise to a secondary type zoospore and thus shows dimorphism (i.e. diplanetism) and not merely repeated zoospore emergence as stated by Fitzpatrick (1930). Unfortunately we could not obtain a reasonable number of empty cysts in either species. Neither aeration nor variation of temperature enhanced germination of the primary cysts. The full primary cysts examined seemed smooth apart from a tuft of radiately arranged hairs seen occasionally.

Achlya

A. bisexualis Coker (male), *A. flagellata* Coker, and *A. racemosa* Hildebrand were obtained from C.B.S. *A. hypogyna* (?) Coker and Pemberton was isolated from a stream near Sheffield. The genus is mainly characterized by the behaviour of the escaping primary zoospores, which encyst at the mouth of the sporangium and form there a more or less spherical aggregation. Their failure to separate has been explained in various ways. Hartog (1888) wrote of 'a mutual attraction of the spores' which he terms adelphotaxy, whilst Rothert (1888) demonstrated protoplasmic connexions between the spores on emergence, and was of the opinion that the spores are held together by mucilage. Couch (in Coker, 1923) confirmed the presence of protoplasmic connexions between the spores of *Achlya*. Recently Bonner (1952) has inferred the presence of a 'gummy adhesive' attaching the cysts to each other. The electron microscope revealed that the primary cysts in *Achlya* are completely smooth. No evidence of material connecting the cysts was obtained. Fig. 11 shows two full and some empty cysts from the mouth of the sporangium in *A. hypogyna* and Fig. 12 shows a similar cyst in *A. bisexualis*. The secondary cysts in both species bear short simple hairs without hooks (Fig. 13). In *A. flagellata* and *A. racemosa* it seems that the secondary cysts are smooth, like the primary cysts.

Protoachlya

P. paradoxa Coker. Although *Protoachlya* resembles *Saprolegnia* in its asexual behaviour, the electron microscope shows that the cysts are like those of *Achlya*. Both types of cyst are completely smooth.

B. THE MONOPLANETIC GENERA

In these forms the primary zoospores encyst within the sporangium and the resulting primary cysts germinate to release the secondary zoospores, which are thus the first swimming spores. Encystment of these zoospores may be followed by further motile phases due to repeated zoospore emergence. Two groups of genera can be distinguished:

1. *Thraustotheca*, *Calyptralegnia*, and *Brevilegnia*: the primary cysts formed within the sporangia are released by 'irregular rupture or disintegration of the sporangium wall' in *Thraustotheca* (Salvin, 1942) and by dehiscence of an apical cap in *Calyptralegnia* (Coker, 1927). Salvin (1942) has claimed that *Brevilegnia* can no longer be separated from *Thraustotheca*, and has transferred species of the former genus to *Thraustotheca*.
2. *Dictyuchus*: here the primary cysts within the sporangium germinate *in situ*, each cyst releasing a secondary zoospore through a separate papilla in the sporangial wall.

Thraustotheca

T. clavata (de Bary) Humphrey. The primary cysts which are set free by disintegration of the sporangial wall are quite smooth. Unfortunately we have so far been unable to obtain sufficient empty secondary cysts to examine them.

Calyptralegnia

C. achlyoides Coker & Couch. In this species we have again been able only to examine primary cysts, which are completely smooth.

Brevilegnia

Among four species investigated only *B. unisperma* Coker & Braxton var. *delica* Coker produced zoospores. The primary cysts are smooth. The few available preparations of secondary cysts suggest that these are also smooth.

Dictyuchus

D. sterile Coker proved very suitable since repeated zoospore emergence took place readily from the secondary cysts, thus yielding ample material of empty cysts suitable for examination. The structure of these cysts is unique. During encystment the secondary-type zoospores develop large simple spines. Fig. 15 shows a spore after encystment, whilst Fig. 14 shows the empty cysts. Their whole surface is regularly covered with spines, giving the appearance of a horse-chestnut fruit. Whether these structures have any significance in the biology of *D. sterile* is open to conjecture. An investigation of further species of *Dictyuchus* is clearly desirable in order to discover whether these structures are characteristic of the genus.

DISCUSSION

Although these studies are incomplete they suggest that cyst structure might serve as a useful taxonomic criterion. As Moreau and Moreau (1938) write: 'On ne peut qu'être frappé de la place que les systématiciens accordent aux caractères fluctuants chez les Saprolégniées aussi bien pour la détermination des genres que de celle des espèces.' It may be that in the cyst membranes a more constant generic character has been found.

Whilst it is clearly desirable to study more species, the structures of the cysts of *Saprolegnia ferax* and *S. dioica* (representative of the 'ferax' and 'diclina' groups of the genus) are so similar as to suggest that they might characterize the genus as a whole. The close similarity of the cysts of *Isoachlya eccentrica* and *I. unispora* to those of *S. ferax* and *S. dioica* suggests a close relationship. Coker (1923) writes: 'The genus (*Isoachlya*) is characterized and distinguished, in the main, by the presence of a cymose or *Achlya* mode of formation of the secondary sporangia, coupled with diplanetic spores.' It is unfortunate that *I. toruloides* (the type species) was not available for examination. Study of these species may enable a reassessment of the genus, and may fail to uphold its distinction from *Saprolegnia*.

S. parasitica differs from *S. ferax* and *S. dioica*. However, the hooks in the former species are clearly similar to those of the latter (they may be thought of as longer variants arranged in groups), and may perhaps be regarded as an adaptation to a parasitic existence. It seems reasonable to suppose that the hooks in *Saprolegnia* and *Isoachlya* may assist in attaching the secondary cysts to the substratum, but whether their absence from other genera indicates differences in biology must remain open to conjecture until more precise information is available concerning the actual substrata colonized by the secondary cysts.

The structure of the cysts in *Protoachlya* clearly separates it from *Saprolegnia* and *Isoachlya*, and suggests a closer relation with *Achlya*.

It is impossible to make any conclusions concerning the monoplanetic genera, since studies on the secondary cysts of most genera are still lacking. The observations on the diplanetic forms suggest that it is the secondary cysts which are more likely to yield critical information.

The chemical nature of the cyst ornamentation is uncertain, but we suspect that the hooks do not consist of the same material as the cyst walls, i.e. cellulose (Frey, 1950). The disintegration of the cyst wall occurs without breakdown of the hooks. The simple hairs found on various cysts may be cellulose fibrils projecting from the cyst wall, although it is possible that all these structures are not homologous. The spines of *Dictyuchus* seem to be merely outpushings of the cyst.

We have little to add to the excellent description of Rothert (1894) concerning the fate of the flagella during encystment. Rothert gives clear evidence that the secondary zoospores simply shed their flagella. This can be confirmed by mounting motile secondary zoospores on to a grid and leaving them to encyst there. Detached flagella of both types, about equal in number to encysted spores, are then found. When the primary zoospores encyst Rothert has shown that the flagella are not shed, but that they are resorbed into the body of the spore. He states that he could never observe a part of the flagellum remaining outside the primary spore after encystment. Although Rothert is probably correct in stating that the flagella are resorbed, he may be wrong in stating that no part of them remains outside the cyst. In our opinion the radiately arranged tufts of hairs, found on or near the primary cysts in several genera (Figs. 2, 5, 8) are probably the remains of the absorbed ciliated flagella. This conclusion is supported by the fact that such structures were only found on primary cysts and also that the radiating hairs are about the same length as the *Flimmer*-hairs of the flagella. Further, the original photographs of Figs. 5 and 8 show that the extremities of the radiate hairs extend into finer processes which may also be seen at the tips of the *Flimmer*-hairs in the original photographs of Fig. 6. This thinning of the apices of the *Flimmer*-hairs has already been fully described in *Saprolegnia ferax* by Manton *et al.* (1951), and is illustrated in Fig. 8 of their paper.

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DESCRIPTION OF PLATES

PLATE I

Fig. 1. *Saprolegnia dioica.* Four cysts: two with hooks, two without. × 6,600.

Fig. 2. *S. ferax.* Primary cysts with small single hairs and a radiate tuft. × 8,300.

Fig. 3. *S. ferax.* Secondary cysts with hooks. The framework of cellulose fibrils in the wall is visible. × 8,300.

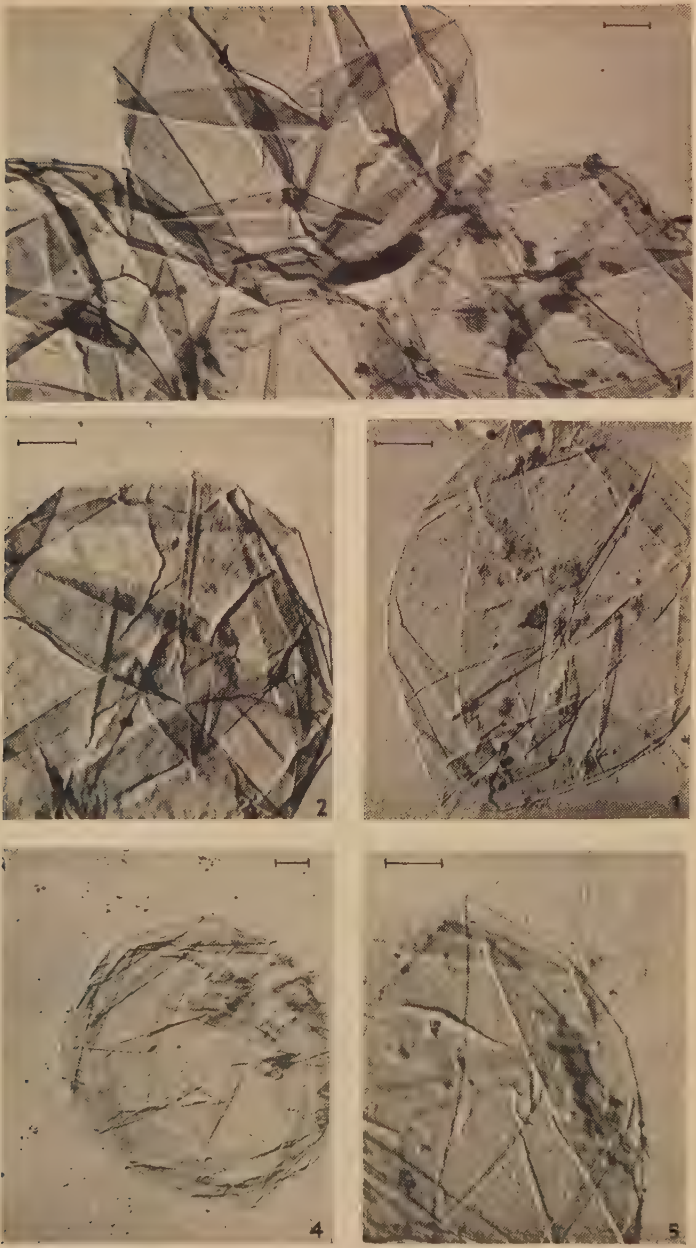
Fig. 4. *S. parasitica.* Primary cyst with long single hairs, but no hooks. × 5,000.

Fig. 5. *S. parasitica.* As in Fig. 4, but with radiate tuft of hairs. × 8,300.

PLATE II

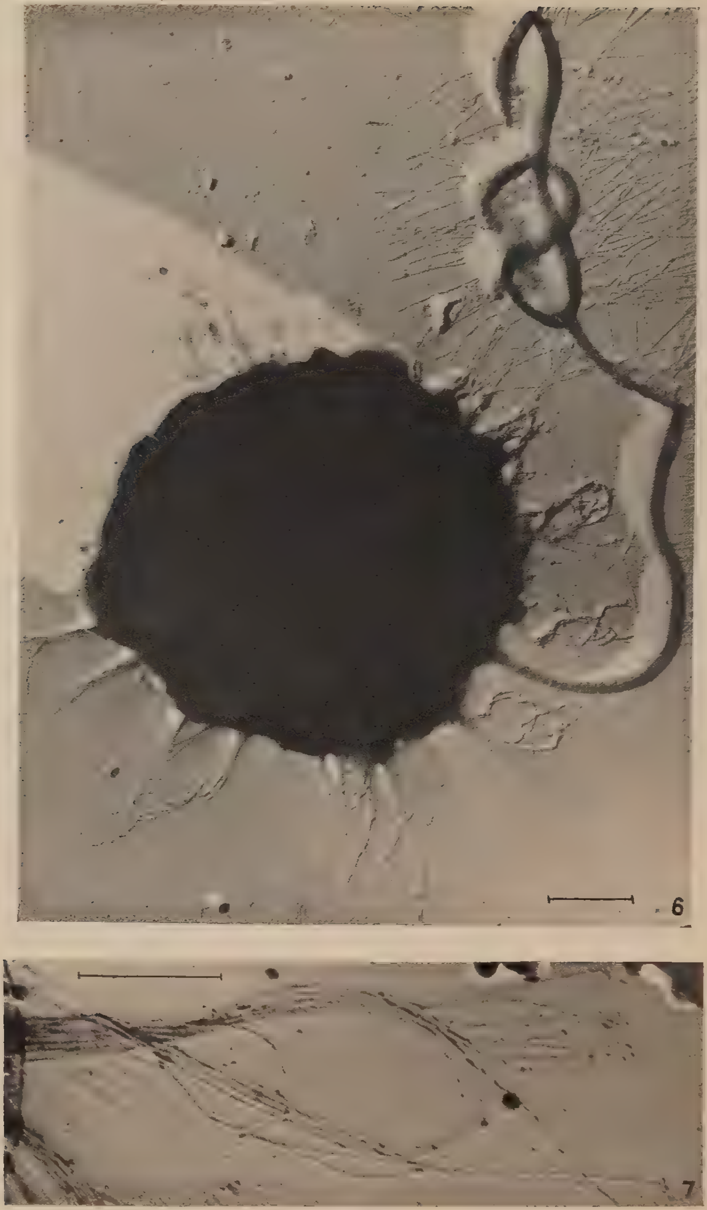
Fig. 6. *Saprolegnia parasitica.* Secondary zoospore in the phase of encystment with a ciliated flagellum still attached. Tufts of hooked hairs are being formed. × 12,500.

Fig. 7. *S. parasitica.* Single tuft of hooked hairs from a secondary cyst. Some of the stalks are larger than the others and are bent backwards. × 21,000.



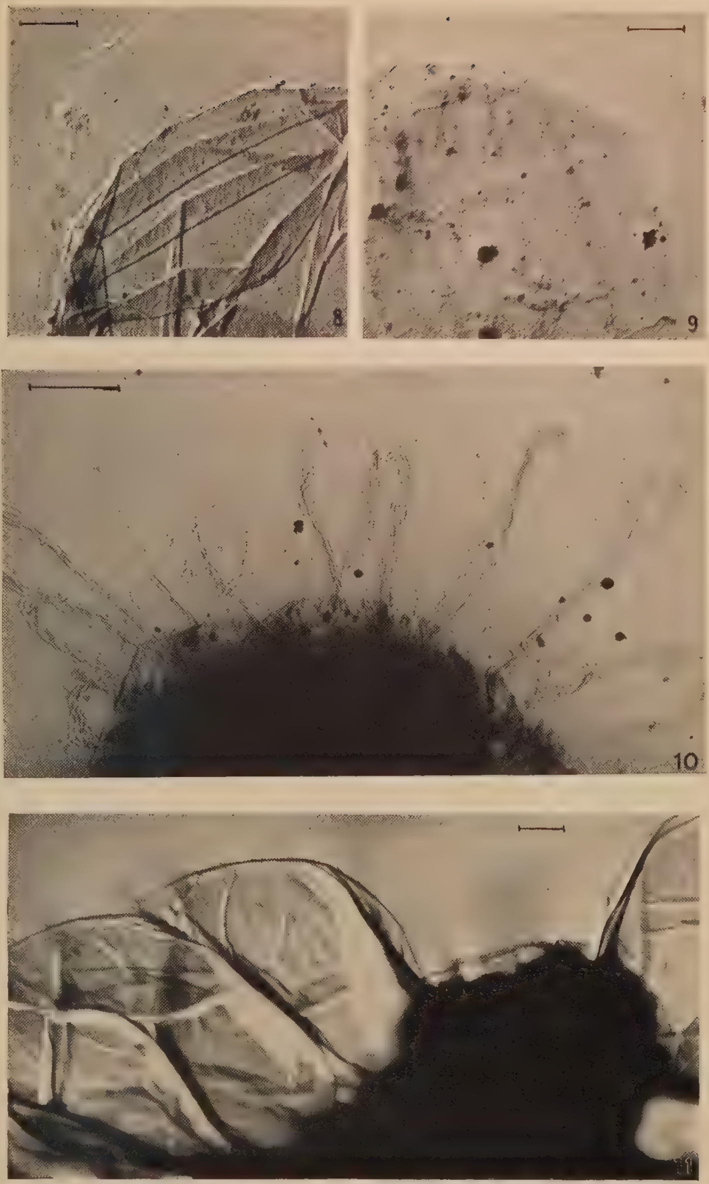
Figs. 1–5

H. MEIER *and* J. WEBSTER—PLATE I



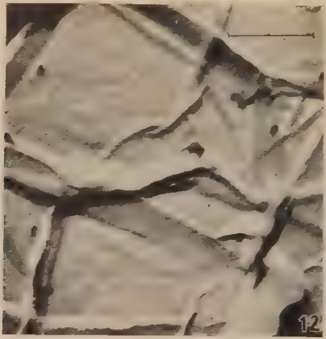
Figs. 6, 7

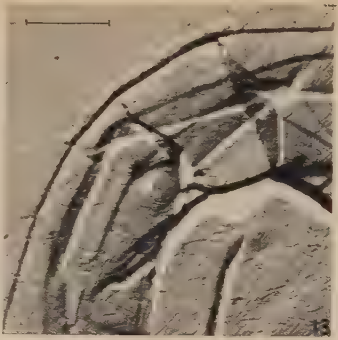
H. MEIER *and* J. WEBSTER—PLATE II

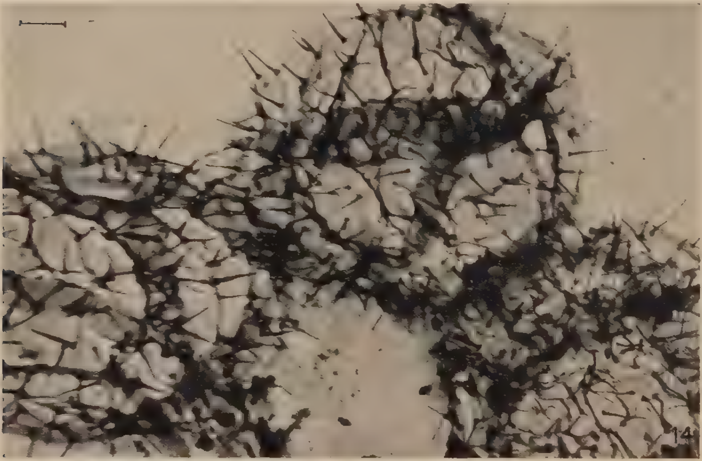


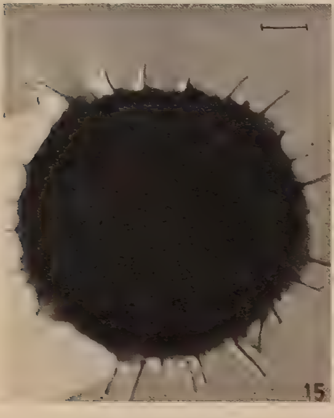
FIGS. 8–11

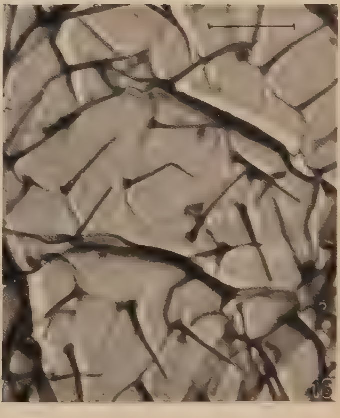
H. MEIER *and* J. WEBSTER—PLATE III











FIGS. 12–16

H. MEIER *and* J. WEBSTER—PLATE IV

PLATE III

Fig. 8. *Isoachlya unispora.* Primary cyst with short hairs and a radiate tuft of hairs. × 8,300.

Fig. 9. *I. eccentrica.* Secondary cyst with hooks. × 8,300.

Fig. 10. *S. parasitica.* Part of a full secondary cyst with tufts of hooked hairs, most of which are bent backwards. × 12,500.

Fig. 11. *Achlya hypogyna.* Primary cysts from the mouth of a sporangium. Two are full, others are empty. × 6,600.

PLATE IV

Fig. 12. *Achlya bisexualis.* Empty cyst from the mouth of a sporangium. × 12,500.

Fig. 13. *A. bisexualis.* Secondary cyst with short hairs. × 12,500.

Fig. 14. *Dictyuchus sterile.* Three empty cysts with spines. × 6,600.

Fig. 15. *D. sterile.* Full cyst with spines. × 6,600.

Fig. 16. *D. sterile.* Higher magnification of a part of a cyst. × 12,500.

An Electron-microscopic Investigation of the Walls of Conifer Cambium

R. D. PRESTON AND G. W. RIPLEY

Botany Department, University of Leeds

WITH 2 PLATES

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SUMMARY

Electron-micrographs of the primary walls of conifer cambium reveal the type of structure already found in other primary walls, except that no area of 'loose' microfibrils appear to be present. In addition, groups of fibrils are visible radiating from a centre which appears to be granular and recalling strongly the putative islands of synthesis reported in *Valonia*.

INTRODUCTION

THE nature of the growing cell wall has been the subject of inquiry for a number of years and the results of electron-microscopic studies have proved particularly intriguing. Like the secondary walls, primary walls consist of cellulose microfibrils which, however, tend to lie almost transversely to cell length, with considerable angular dispersion about this preferred orientation (Frey-Wyssling *et al.* 1948; Muhlethaler, 1950). In addition, the microfibrils twist around each other (Frey-Wyssling, 1948), as they are also known to do in the adult wall of, for instance, the vesicles of *Valonia* (Preston and Kuyper, 1951). This is taken to constitute one further piece of evidence in favour of the view that these primary walls are permeated by cytoplasm and represent part of the living system rather than dead envelopes. The work reported up to the present, apart from observations on the growth zone of *Phycomyces* (Roelofsen, 1949; Middlebrook and Preston, 1952), refers to the primary walls of parenchyma and fibres. During an investigation planned to attempt a determination of the structure of microfibrils, one of the experimental objects has been the cambium of conifers. It has been thought worth while to present the observations made on this tissue because, in the first place, no electron-microscopic investigation of a lateral meristem has yet appeared. In addition, an observation has been made which, though difficult to interpret in itself, resembles so closely the earlier observations concerning microfibril synthesis (Preston and Kuyper, 1951; Preston, Nicolai, and Kuyper, 1953) that it seems worth while to put it on record.

The fusiform initials of the cambium, particularly of conifer cambium, have been the subject of much investigation from the point of view of wall

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structure, both under the polarizing microscope and in the X-ray spectrometer. It may be recalled that these cells are usually very elongated, the length: breadth ratio ranging up to 100 or more. Apart from the temporary seasonal cessation of activity they are capable of continual elongation over many years. During the growing season these cells divide frequently by longitudinal tangential division, cutting off on the inside cells which subsequently differentiate into elements of the wood. The thin primary walls are able therefore to increase both in length and breadth. The walls contain in cellulose, which can be recognized by its characteristic X-ray diffraction diagram (Preston, 1946), clearly associated with pectins and other non-cellulosic substances (Preston and Wardrop, 1949). This primary wall, now somewhat changed chemically, still lies on the outside of a mature tracheide in the wood and here it has been shown that the molecular chains of cellulose lie at an angle of about 16° to the transverse plane (Preston, 1947). In the cambium itself the available X-ray data are compatible with a similar orientation (Preston and Wardrop, 1949). The primary walls of the cambium resemble therefore those of other cell types in being wound with molecular chains of cellulose lying almost transversely to cell length.

MATERIALS AND METHODS

Cambial tissues have been examined from three species –*Pseudotsuga taxifolia*, *Larix leptolepis*, and *Populus* sp. The material was collected immediately after the onset of cambial activity in the spring of 1953, by the stripping method usual in this laboratory. Immediately after removal from the tree the strips were placed in 5 per cent. formalin and transferred within an hour to 50 per cent. alcohol. They remained in the alcohol until required.

Examination under both an ordinary and a polarizing microscope showed that no noticeable differentiation had taken place, so that the wall was still substantially primary.

In preparing specimens for examination the strips were washed repeatedly in distilled water and homogenized in a 10-ml. Nelco homogenizer until the solution became opalescent. It was then allowed to stand and both the 'precipitate' and the supernatant fluid were examined.

RESULTS

Essentially the same observations were made on all three species, but for reasons of space only those electron-micrographs are presented which illustrate most clearly the typical features of these cambial strips (Plate I, Figs. 1–3; Plate II, fig. 4).

Fig. 1 shows the general appearance of material taken from the precipitate. It seems very probable that this represents the intact primary wall with a minimum of disturbance due to homogenization. The wall is clearly composed of a meshwork of intertwining microfibrils embedded in some amorphous material. It is impossible from such photographs to estimate the diameter of the microfibrils, but our general knowledge of the wall and of the morphology of cellulose would seem to make it certain that the microfibrils are cellulosic; and the amorphous material therefore of a pectic and perhaps protein nature.

The microfibrils are seen more clearly at a torn edge (Fig. 2). They appear to be rather more than 100 Å in diameter and are therefore much narrower than the microfibrils observed in the algae (Preston *et al.*, 1948; Preston, 1951), but of the same order as those in the secondary walls of the elements of wood (Ranby, 1949; Preston and Ripley, *unpub.*). As reported in the algae, these microfibrils appear to be flat ribbons rather than cylinders. They tend to be oriented in a preferred direction, with considerable angular dispersion, as already concluded from the results of X-ray analysis. A common feature of the wall fragments found in the precipitate is illustrated in Fig. 3 in the form of groups of small perforations. These appear with such regularity and are so stable in the electron beam that they are probably integral parts of the wall architecture. They recall very closely the electron-micrographs of the primary walls already published and are very probably of the same nature, i.e. groups of plasmodesmata. The largest perforation visible in Fig. 3 is about 0·2 μ in diameter.

By far the most intriguing observations were made, however, when drops of the supernatant liquid were dried on the slide. Apart from frequent thin pieces of apparently intact wall resembling very closely that illustrated in Fig. 1, all three species repeatedly yielded a material represented by Plate II, Fig. 4. This gives strongly the impression of microfibrils radiating from centres and manifests microfibril ends which have not hitherto been seen by us in any cellulose except when chemically treated. It is noteworthy that the centres from which the microfibrils appear to radiate seem to be granular, and that amorphous material appears to be missing from among the microfibrils. It is not, of course, clear how far this latter feature is due to the treatment the material has received.

DISCUSSION

There is little need to discuss the general observations on the intact wall. They resemble those made by earlier authors already quoted and support their statements concerning both orientation of the microfibrils and their frequent twisting round each other. Most other publications refer, however, to a localized loosening of the structure of the wall either in randomly arranged areas or at certain restricted regions such as the tips of elongating cells. It is in these looser regions that wall synthesis is thought to be occurring and by means of them that growth in area is achieved. The present investigation has yielded no signs of such phenomena except under conditions in which it was quite obvious that the loosening was an artefact arising, presumably, from mechanical damage during homogenization [as can readily be observed even in homogenized secondary wall material]. This absence of looser areas cannot be due to a localization of growth at the tips of the cells, which might otherwise seldom be observed, for the major extension in wall area of cambial cells

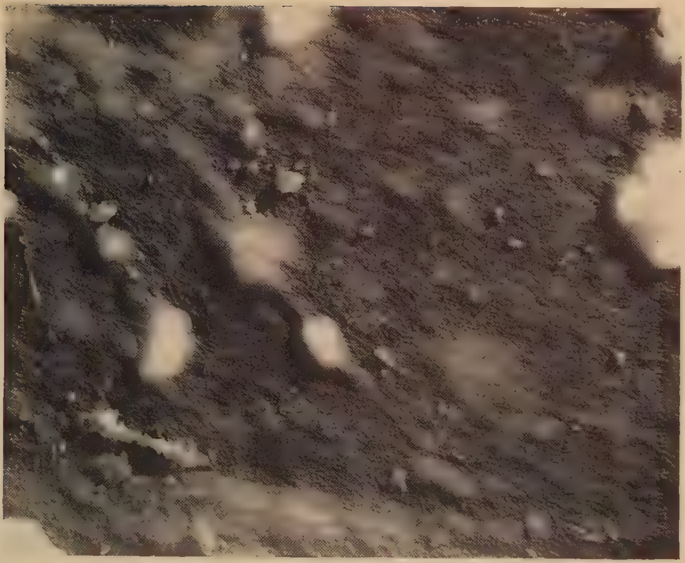

FIG. 1

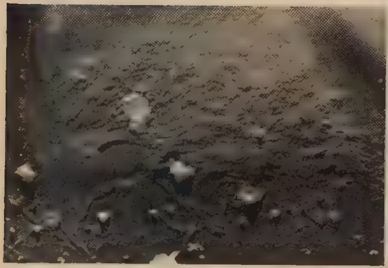

FIG. 2

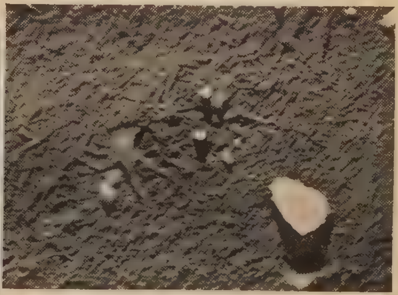

FIG. 3

All the illustrations are electron micrographs of pieces of the cell walls of conifer cambium shadowed with Pd–Au.

FIG. 1. Cambium of *Populus* sp. Note the fine, apparently intertwined microfibrils. Magnification ×22,200

FIG. 2. Cambium of *Pseudotsuga taxifolia*. Note the prominent microfibrils appearing at the torn edge. Magnification ×22,200

FIG. 3. Cambium of *Larix leptolepis*. Magnification ×22,200

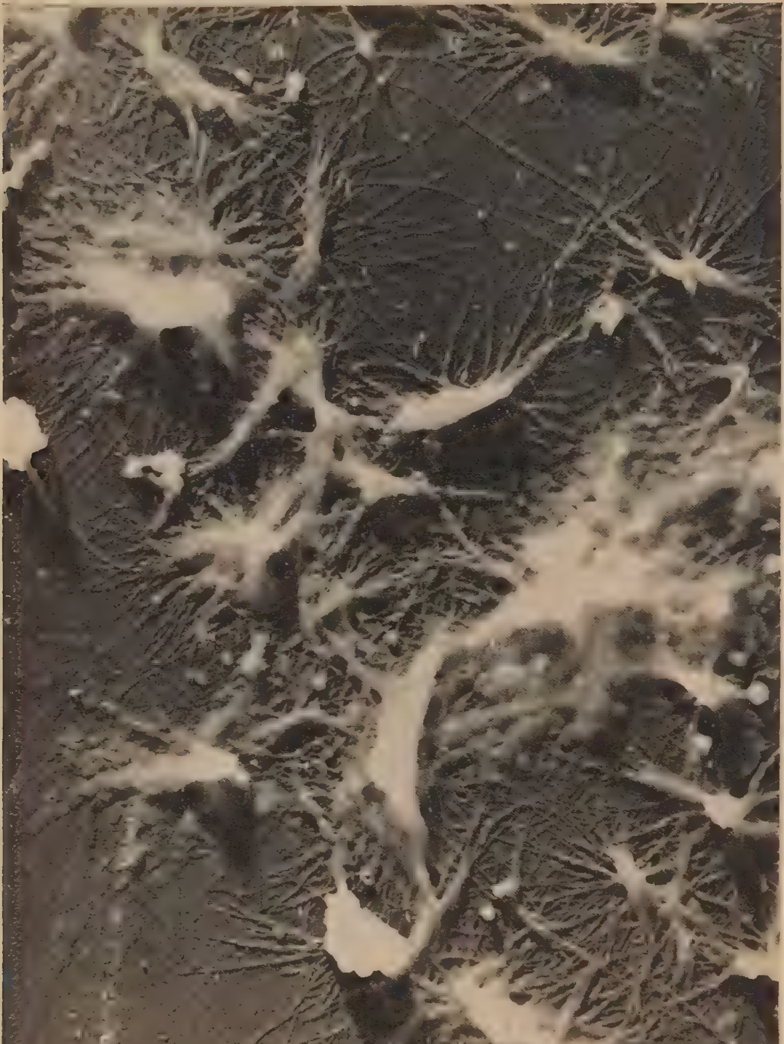

FIG. 4. Typical appearance of material from the supernatant liquid when the cambium of *Populus* is treated in a blender. Note the numerous fibril ends and that the microfibrils appear to radiate from centres. Magnification ×20,600

occurs transversely. Nor can it easily be due to the fact that only radial walls increase in area, for the chances of breaking fragments from radial and tangential walls would seem to be equal. It could be, of course, that the cambial cells, at the moment of collection, had only just swollen and not yet commenced growth, but this we can hardly believe. The absence of looser areas is thus somewhat intriguing in view of the repeated observations made in other primary walls. It is impossible at the moment to draw any conclusions from this; it may be recorded only that there is as yet no evidence of intussusception as a factor in the growth of cambial cell walls. Some significance may perhaps be attached to the consideration that these are the first walls to be examined in which extension in wall area occurs transversely, i.e. approximately parallel to the preferred direction of the cellulose microfibrils.

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Respiratory Responses in Potato Tubers as an Index of the effect of Ethylene Chlorhydrin and Ammonium Thiocyanate

SHRI RANJAN AND RAVINDAR KAUR

Botany Department, University of Allahabad (India)

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SUMMARY

1. The variety 'Phulwa' was worked upon for respiratory studies and growth response to dormancy-breaking hormones. The hormones were used in single and double doses.

2. The respiratory studies reveal that ethylene chlorhydrin affects only the bud areas and not the non-bud areas.

3. The intensity of the effect of the hormone progressively decreases in the deeper layers of cells, till finally no effect is visible in the cells of the pith areas.

4. A distinct correlation exists between the respiratory intensity and dormancy.

5. Of the two hormones used the respiratory rates recorded for N. ethylene chlorhydrin treated tubers, given in single and double doses, were the highest. Similarly the growth response to both the doses showed the maximum reduction of the dormancy period. 0·1 N. ethylene chlorhydrin treatments were not effective in breaking the dormancy or in significantly altering the rate of respiration.

6. Treatments with 0·5 per cent. ammonium thiocyanate given in single dose showed the lowest respiratory rates and the germination records showed no difference over the control. With the double dose a gradual rise in the respiratory rates was observed. The germination records also showed an earliness of 20 and 22 days over the control and single-dose treatments respectively.

7. Treatments with 2 per cent. ammonium thiocyanate depressed germination. The respiratory rates recorded were also low.

8. In general the double-dose treatments showed better results than the single-dose treatments. Also, treatments on 3 weeks old tubers were more effective than on 6 weeks old tubers.

BREAKING the dormancy is as important a problem as prolonging the dormancy of potato tubers. This is specially so for a vast country like India with its special problems. The province of Uttar Pradesh, which is the largest potato-growing State, grows 90 per cent. of the total crop produced in India. The sowing season in the plains is from August to October, when such varieties as 'Phulwa', 'Gola', &c., are generally sown. But the better table varieties like 'Majestic' and 'Up-to-date' are grown only on a very restricted scale. Usually the sowing season of these varieties is from mid-November to the first week of January. These are harvested from March to the end of April.

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April, being a very hot month in the plains of India, has an adverse effect on the sprouting of tubers.

'Up-to-date' or 'Majestic', which are hill varieties, when sown in the plains have relatively shorter dormancy periods than 'Gola' or 'Phulwa', the plain varieties. Therefore this problem of shortening the dormancy may not affect them. But in Uttar Pradesh these hill varieties are brought down to the plains from September onwards as freshly harvested potatoes. These do not germinate till November or December. Here the problem of breaking the dormancy arises.

Secondly, blight, which is relatively less destructive when potatoes are grown in the cooler regions of the hills, does havoc to the seed tubers when they are transported from the hills and sown in the plains. Therefore, if by suitable hormone treatment the dormancy of these tubers is broken earlier, and if these could be sown in October rather than in December, they would escape the hot dry period of April. Also, the tubers could be stored in the plains and when used for seed purposes these acclimatized tubers would be less liable to fungal attacks.

Freshly harvested potato tubers if replanted in favourable growth conditions do not sprout at once but require a period of dormancy. Denny, Guthrie, Miller, and many others have recommended various chemicals for breaking the dormancy of freshly harvested tubers. One of the chief hurdles in this type of experiment is to find out the correct dosage of the hormone required for reducing the dormancy of potatoes. According to Denny the problem is to devise a method of assuring oneself within a day or, at the most, within 5 days after treating the tubers that the treated tubers have been brought into the desired condition. One of the methods suggested by Denny is to estimate the amount of hormone vapours absorbed by the potatoes when the hormone in question is enclosed in the jar with the potatoes. According to him 90 per cent. sprouting took place within 30 days from planting when 8 to 10 ml. of 0·1 N. ethylene chlorhydrin was absorbed per 100 ml. of pressed juice. The method is rather tedious. In this paper a convenient method of testing the efficacy of the hormone by studying the respiratory activity of eyes of the treated tubers is described.

EXPERIMENTAL PROCEDURE

Materials. For these experiments the variety 'Phulwa', which is a common plain variety, was selected. The tubers were purchased from the Agricultural Institute, Allahabad.

Hormones: Ethylene chlorhydrin was applied at concentrations of 20 and 10 ml. N. and 0·1 N. per 100 g. tissue in the single-dose treatments. In double-dose treatments 20 ml./100 gr. tissue were used. 0·5 and 2·0 per cent. ammonium thiocyanate were used in both single- and double-dose treatments. These substances are referred to here for conciseness as 'hormones'.

Methods. Vapour and soak methods were used for the hormone treatments and Warburg manometric methods for respiratory studies.

Experimental procedure: Respiratory studies

In the single-dose treatment, four lots of the variety 'Phulwa', which were harvested on 5 February 1952, were selected. The tubers were treated on 26 February 1952. Each lot consisted of four potatoes of uniform size and shape, weighing approximately 200 g. They were kept in air-tight jars of 1 gallon capacity. Ethylene chlorhydrin, of N. and 0·1 N. concentrations, was kept in two small Petri dishes in which cloth was crumpled to increase the evaporating surface. Petri dishes with water were kept in the control jars. After 24 hours of this vapour treatment one potato of each set was taken out for experimental purposes. The rest of them were removed from the jars. They were placed in their respective paper bags at room temperature (31° C.), to be experimented upon for respiratory studies on subsequent days.

Two eyes from each potato were taken with an 8-mm. cork borer and sliced off to a uniform length of 5 mm. each, weighing roughly 300 mg. Respiration was then measured in the Warburg apparatus. In the double-dose treatment the variety 'Phulwa', harvested on 4 March 1952, was treated on 30 March in the same way but the tubers were given a second dose of the hormone 4 days after the first treatment. Tubers treated with ethylene chlorhydrin (Ecl) were removed from the jars after 24 hours and divided into two lots of three tubers each. The first lot of tubers was placed in paper bags for respiratory studies of the first dose of hormone. The second lot of tubers was also placed in paper bags and kept aside at room temperature (33° C.); these were then exposed to the vapours of Ecl again 4 days after the first treatment, i.e. on 3 April. Twenty-four hours later the tubers were removed from the jars and placed in paper bags, from which they were taken out periodically for subsequent respiration studies.

Ammonium thiocyanate treatment consisted in soaking six tubers in 0·5 and 2·0 per cent. solutions for 1 hour each. The tubers were taken out of the solutions after an hour and divided into two lots of three tubers each. The first lot of tubers was placed in paper bags for respiratory studies of the first dose of the hormone. The second set of tubers was stored in paper bags, and the tubers were soaked once again in these two ammonium thiocyanate solutions 4 days after the first treatment. After a period of 1 hour the tubers were placed in paper bags for subsequent respiratory studies.

Respiratory studies of bud and non-bud areas of treated and untreated tubers at various depths. The main purpose of conducting these experiments was to find out whether (*a*) the hormone affects only the bud areas or whether there was a general effect on non-bud areas as well, and (*b*) whether it affects only the superficial layers of cells or its effect penetrates into the deeper layers.

For the bud and non-bud areas the experimental procedure was the same as already described for the respiratory studies. In all cases similar pieces of untreated tubers were used as control. Respiration was measured on the first, second, and fourth days of the treatment in all cases.

Growth studies. In the single-dose treatment potatoes harvested on 15

January and on 5 February 1952, i.e. after 6 and 3 weeks of storage, were treated with ethylene chlorhydrin and ammonium thiocyanate as described. The method of hormone application was the same as in the case of respiratory studies. After 24 hours' treatment with ethylene chlorhydrin the tubers were placed in paper bags and stored for 6 days at room temperature (31° C.). Thereafter they were sown in pots, three in each.

In the case of ammonium thiocyanate twelve tubers were dipped in 0·5, 1, and 2 per cent. solutions of this hormone for 1 hour, after which they were removed and planted immediately without being rinsed. There were four replications of each of the above treatments. The pots were kept at room temperature (31° C.) and were watered at periodic intervals.

The double-dose treatment followed an experimental procedure similar to that used in respiratory studies.

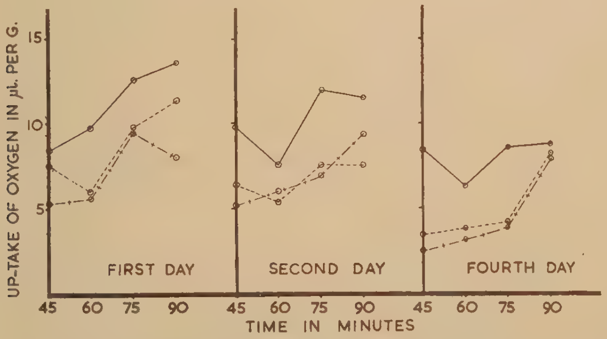

FIG. 1. Respiration of 'Phulwa' potato excised buds, 1, 2, and 4 days after single-dose treatment with N. ethylene chlorhydrin (—), 0·1 N. ethylene chlorhydrin (– – – –). Controls are shown (– + – + –).

RESULTS

Respiration. Single-dose treatment with ethylene chlorhydrin caused changes in respiration rate of excised buds which are shown in Fig. 1. There is a tendency for the respiration to drift upwards after cutting. Further, on the first, second, and fourth days after treatments, those given N. ethylene chlorhydrin showed significantly higher respiration than those with 0·1 N. and control.

Double-dose treatment also caused changes which are not given in detail in respiration rates of the eyes. N. ethylene chlorhydrin again caused increase, but the treatments with 0·1 N. ethylene chlorhydrin, 0·5 and 2 per cent. ammonium thiocyanate did not produce significant effect.

Respiratory studies of bud and non-bud areas at varying depths of treated and untreated tubers. Fig. 2 shows respiration rates of different regions in treated and untreated tubers. Treatment affects surface and cortical layers markedly in the bud area and is ineffective in non-bud areas. Lack of effect on pith cells may indicate lack of penetration of hormone to this depth.

Growth response: Single-dose treatment. Tables I and II show the chemical treatments given, the date of visible sprouting, days required for 90 per cent. sprouting, and percentage of tubers which rotted. It will be seen from these tables that ethylene chlorhydrin (20 ml. N.Ecl/100 g. tissue) is the most effective dormancy-breaking treatment, followed closely by half this dosage and by 1 per cent. ammonium thiocyanate. 0·1 N. ethylene chlorhydrin was little different from the controls.

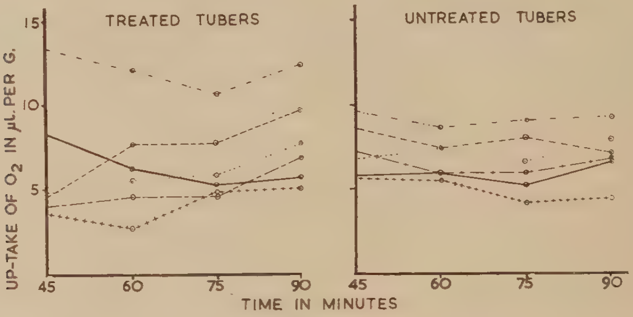

Fig. 2. Respiration of different zones of treated and control tubers. Bud area epidermis (— · · —), cortex (—), pith (+ + + +). Non-bud area epidermis (– – – –), cortex (· · · · · ·), pith (– – –).

Considerable rotting of controls and 0·1 N. ethylene chlorhydrin treated tubers is to be noted; other treatments promoted germination and rotting was avoided. Best treatments secured germination 3 weeks before control. Tubers treated 3 weeks after harvesting responded more readily than those treated 6 weeks after harvesting.

DISCUSSION

Miller (1933) studied the effect of chemicals on the respiratory activity of whole potato tubers. He finds that ethylene chlorhydrin, ethylene bromhydrin, hydrogen sulphide, ethyl mercaptan, &c., increase the respiratory rate, but he could not get any correlation between increased respiration and shortening of dormancy.

We have made an attempt to show that a distinct correlation exists between respiratory rates of potato pieces and the shortening of dormancy. Our studies show, furthermore, that as a result of treatment with dormancy-breaking chemicals the greater part of the bulk of the tuber tissue has an unchanged respiration. The changes in respiration associated with such treatment are only found in the relatively superficial regions of the bud areas. The failure to obtain correlation between respiration and dormancy breaking in the past, one may conjecture, has been due to the fact that bulk respiration

TABLE I

Germination of tubers of potato variety 'Phulwa', treated with dormancy-breaking hormones on 26 February 1952

	Treated 6 weeks after harvest			Treated 3 weeks after harvest		
	Date of first sprouting	Days for 90% to sprout	Rottage %	Date of first sprouting	Days for 90% to sprout	Rottage %
Ethylene chlorhydrin						
20 ml. N./100 g. tissue	25 March	40	0	21 March	33	0
10 ml. N./100 g. ,,	29 ,,	43	0	24 ,,	38	0
20 ml. 0·1 N./100 g. ,,	6 April	(49 for 80%)	20	6 April	(48 for 75%)	25
10 ml. 0·1 N./100 g. ,,	10 ,,	(52 for 60%)	40	3 ,,	(40 for 75%)	25
Ammonium thiocyanate						
2 per cent.	4 ,,	48	0	3 ,,	(50 for 80%)	20
1 per cent.	4 ,,	48	0	24 March	35	0
0·5 per cent.	29 March	42	0	27 ,,	39	0
Control	13 April	(54 for 60%)	40	16 April	(56 for 60%)	40

TABLE II

Germination of tubers of potato variety 'Phulwa' following single- and double-dose treatments

	Single dose 30 March			Double dose 3 April		
	Date of first sprouting	Days for 90% to sprout	Rottage %	Days of first sprouting	Days for 90% to sprout	Rottage %
Ethylene chlorhydrin						
N.	3 May	45	0	27 April	38	0
0·1 N.	10 ,,	50	0	7 May	45	0
Ammonium thiocyanate						
2 per cent.	16 ,,	(54 for 80%)	20	20 ,,	(58 for 70%)	30
0·5 per cent.	20 ,,	(62 for 60%)	40	1 ,,	40	0
Control	23 ,,	(60 for 70%)	30	23 ,,	(60 for 70%)	30

of whole tuber tissue has been measured rather than the respiration of those portions which appear to be active. The present study has not attempted to deal with fundamental causes of dormancy breaking. The data show that the behaviour of this important Indian variety of potato is not dissimilar from that described by the Boyce Thompson Institute workers.

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An Endogenous 24-hour Rhythm in the Growth Rate of the *Avena* Coleoptile

N. G. BALL AND INGA J. DYKE

Department of Botany, King's College, London

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SUMMARY

The rates of growth of coleoptiles of intact *Avena* seedlings were studied by means of time-lapse photography, using infra-red radiation.

When the seedlings are germinated in red light and subsequently transferred to darkness, a growth rhythm is established in which the first peak in the growth-rate curve occurs about 16–17 hours after the transfer, and the second peak 24 hours later. When the transfer is made sufficiently early, three peaks may occur before growth ceases. The occurrence of the peaks and the emergence of the primary leaf are independent of one another.

Alteration of the point in the life-history at which the seedlings are transferred from light to darkness changes the times of occurrence of the peaks, but does not affect the period of the rhythm. The incidence of the rhythm shows no correlation with time of day; therefore the rhythm is not due to diurnal changes in external conditions.

Interruption of the dark period by several hours' exposure to red light causes the suppression of a previously induced rhythm and the establishment of a new one which commences at the time the seedlings are restored to darkness. When they are grown under continuous red light no rhythm is induced.

Within the range 16° to 28° C., temperature has little or no effect on the period of the rhythm.

When seedlings of *Triticum* are grown under the same conditions as those which induce a rhythm in *Avena*, no rhythmical variation in the growth rate can be detected.

INTRODUCTION

THE effects of immersing rhizomes of *Aegopodium podagraria* for short periods in solutions of certain growth-regulating substances have been described by Ball (1953). In order to determine whether similar results would be obtained when the same technique was applied to another plant, a further series of experiments was started by one of us (N. G. B.), using *Avena* seedlings instead of the rhizomes. In each of four experiments the curve showing the change in the mean rate of growth of the coleoptiles revealed the existence of a definite rhythm with a period of almost exactly 24 hours. This rhythm was independent of the effects produced by auxin, but since its existence might be a cause of variation in the response to auxin at different times, we decided to postpone temporarily further experiments using growth substances and to study first of all the nature of the rhythm and the conditions under which it occurred.

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MATERIAL AND METHODS

Grains of 'Victory Oats' were husked, soaked in water for 1–2 hours, and germinated in a sloping position on wet filter paper in covered glass dishes under red light at 24–25° C. After a certain length of time, which was altered in different groups of experiments, 20 seedlings were transferred to the plant chamber of the infra-red photographic apparatus described by Bennet-Clark and Ball (1951) and Ball (1953). Since the apparatus was designed to be used with horizontally growing rhizomes and was now used with vertically growing seedlings, it was necessary to turn it through 90° so that it rested on one side. The perforated strip of perspex which had been used to secure the rhizomes in the plant chamber was replaced by another strip in which 20 slots had been cut to receive the *Avena* grains. Leading from each slot was a vertical groove in the side of the strip that was cemented to the back glass of the plant chamber. Each groove thus formed a short tunnel into which the coleoptile could be inserted. The arrangement of the seedlings can be seen in Fig. 1.

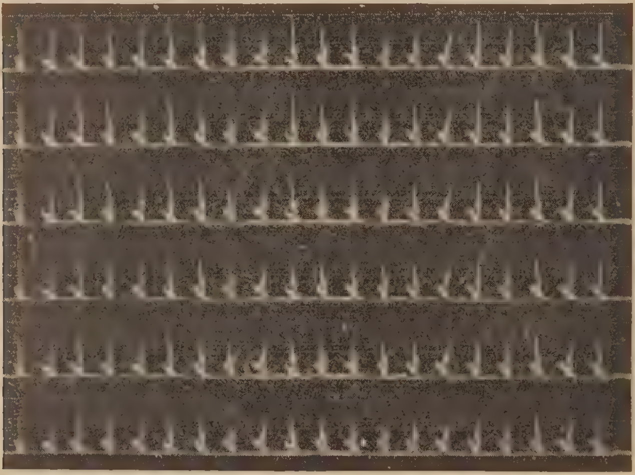

FIG. 1. Six successive photographs, in order from below upwards, of portion of the plant chamber, being part of the record of one experiment (Expt. II), showing the 20 *Avena* seedlings during the period 65–70 hours from soaking. The white horizontal lines in the photographs are 10 mm. apart.

The transference of the seedlings to the plant chamber was carried out under weak orange light. After the plant chamber had been fixed in place in the apparatus the seedlings were in complete darkness except for a short period every hour when they were photographed automatically. They were then exposed for about 1 minute to infra-red radiation, together with the very small amount of red light which passed through the light filters. In these experiments one layer of Ilford filter No. 207 was used together with one of

No. 813. As will be shown later, the effect of this amount of radiation on the seedlings was immaterial. The plant chamber contained large surfaces of wet filter paper and a slow stream of moistened air was passed through it. The whole apparatus was in a basement room in which the temperature was controlled. The range of temperature inside the apparatus during each experiment was recorded on a maximum and minimum thermometer and is shown above the corresponding graph.

Since the photographic recording was carried on for 48 hours or longer, the tips of the coleoptiles soon tended to grow beyond the area included in the photographs. This difficulty was overcome by moving the rising front of the camera from time to time.

The rates of growth were determined by projecting the negatives on to a screen and measuring the positions of the tips of the coleoptiles with reference to the image of the white lines drawn on the black paper fixed to the back of the plant chamber. The mean rate of elongation for each hourly period was plotted. The hour during which each primary leaf emerged from the tip of the coleoptile could easily be determined from the photographs and is recorded below the curve by means of a dot.

EXPERIMENTAL RESULTS

The effect of transferring seedlings from light to darkness. The preliminary experiments, already mentioned, which revealed the existence of a rhythm in the growth rate are not recorded here, but the first three experiments (I, II, and III, Fig. 2) show the same rhythm unaccompanied by the effects of auxin treatment. These experiments were carried out under similar conditions on different occasions, but were started at different times: at 10.30 a.m., 4.30 p.m., and 10.30 p.m. respectively. Each curve shows two distinct peaks, the first 16–17·5 hours after the seedlings were transferred from light to darkness and the second 24 hours later. The closeness of the approximation to a 24-hour period can be judged from the positions of the two vertical dotted lines which have been placed exactly 24 hours apart in these and other graphs. Since the timing of the peaks shows no correlation with time of day, the variation in the growth rate cannot be attributed to the action of diurnal changes in atmospheric or other external factors.

As pointed out previously, from the time the seedlings were removed from red light and transferred to the apparatus they were in complete darkness except during approximately 1 minute every hour when they were exposed to infra-red radiation together with a very small amount of red light. In Expt. IV this radiation was omitted during the first 17 hours after the transfer. Photographic recording was then started and showed that there had been no apparent alteration either in the induction of the rhythm or in its subsequent development. It follows, therefore, that a period of darkness interrupted once every hour by the radiation used in taking the photographs is not materially different in its effects from the same period of uninterrupted darkness.

In Expts. I–IV (Fig. 2) the seedlings were transferred from light to darkness at the 50th hour from soaking. In Expts. V and VI (Fig. 3) this was done at the 45th hour. A comparison of the graphs will show that in each case the first peak is about 16 hours after the transfer, so that in Expts. V and VI this peak appears some 5 hours earlier in the life-history. In VII and VIII, which are replicate experiments where the transfer was delayed until the seedlings were 56 hours old, the peaks in the curves have shifted to the right, although not quite to the extent which might have been expected, since the interval between the commencement of darkness and the first peak is only about 15 hours. Both this discrepancy and the difference in the general pattern of the curves VII and VIII from that of the previous experiments seem open to explanation. If a growth rhythm had not been induced, then the growth-rate curve of the coleoptile would have been of the normal grand period type. If

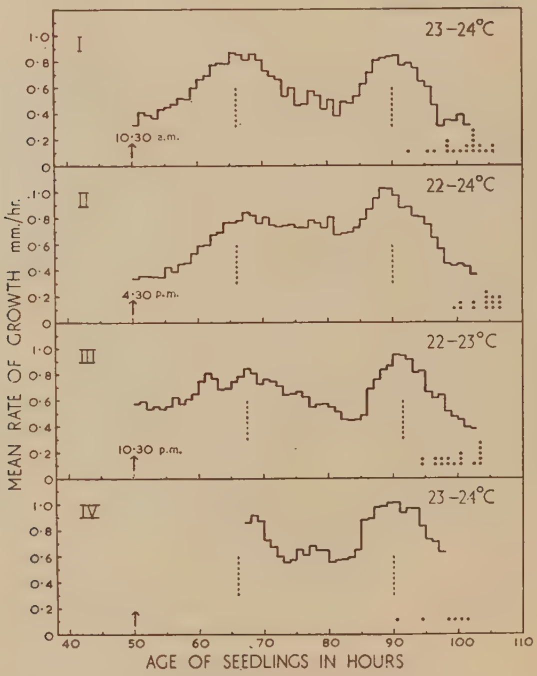

Fig. 2. Graphs I, II, and III show mean rates of growth of the coleoptiles in three experiments started at different times of the day. The age of the seedlings when transferred from red light to darkness is indicated by vertical arrows. The vertical dotted lines are exactly 24 hours apart. Times of emergence of primary leaves are shown by dots. IV is similar, but the infra-red radiation was omitted until 17 hours after the transfer. For further details see text.

one postulates that the growth rhythm induced by the change from light to darkness is superposed on such a grand period curve controlled by other factors, then the form of the resultant curve should depend on the relative positions of the peaks. Assuming the peak of the grand period to be at about 70 hours—which corresponds to the peak of the curve when the seedlings are grown under continuous red light (see Expt. XVI, Fig. 5)—then this could

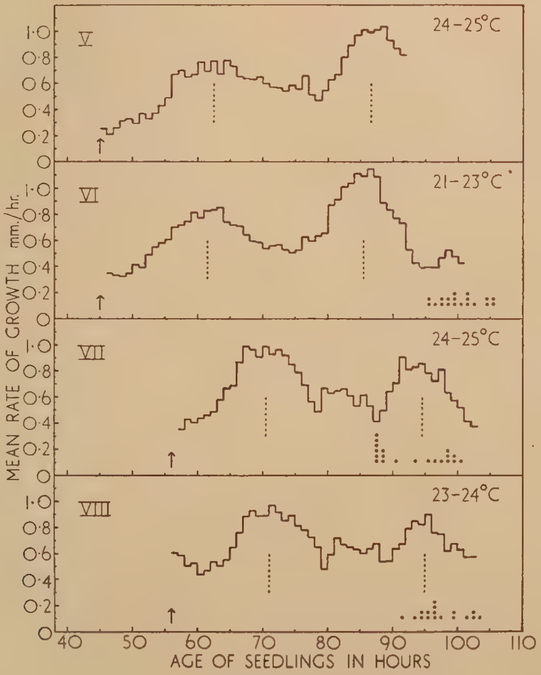

FIG. 3. In V and VI the seedlings were transferred from red light to darkness at the 45th hour. VII and VIII are similar experiments, but the transfer was not made until the 56th hour. Vertical dotted lines are exactly 24 hours apart; times of emergence of primary leaves are shown by dots.

account for the steepening of the curve between 65 and 70 hours in VII and VIII. In the earlier experiments a grand period peak at 70 hours would not occur until just after the first peak induced by the light change and might account for the partial elimination of the depression in this region. In these curves the lowest point is much nearer the second peak, but in VII and VIII, where the grand period peak is assumed to occur before the depression, the latter is more symmetrical and shows two troughs. In both these experiments there are indications of a minor peak midway between the two major ones.

The emergence of the primary leaves in these experiments is recorded by dots just above the time axis of the graphs. (In V, recording unfortunately was terminated before any of the leaves had emerged, and in IV after the emergence of only 6 leaves.) An interesting point is that the emergence of the leaves and the position of the second peak are independent of one another. Where the appearance of this peak is delayed, as in VII and VIII, a high rate of growth of the coleoptile may occur even after the primary leaf has pushed its way through the tip.

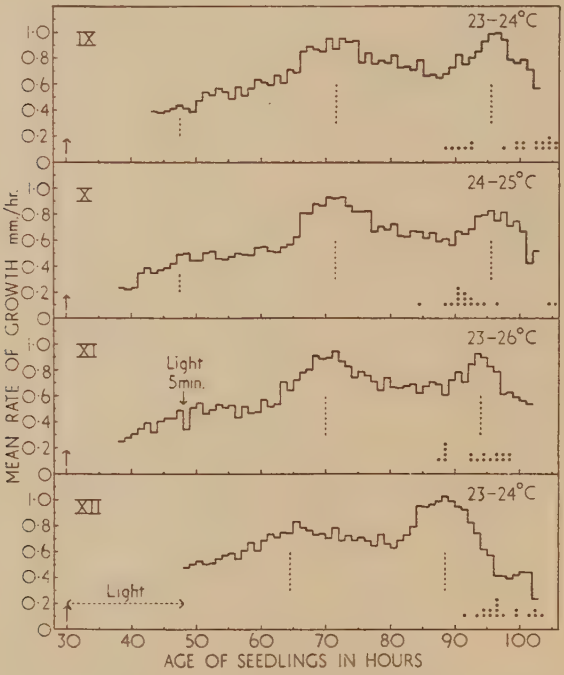

FIG. 4. In IX, X, and XI the seedlings were transferred from red light to darkness at the 30th hour. In XI a 5-minute exposure to orange light was interpolated at the 48th hour. In XII the seedlings were put into the plant chamber at the 30th hour and given a further exposure to red light until the 48th hour, when they were transferred to darkness and recording started. Conventions as in Figs. 2 and 3.

Since the induction of the rhythm took place when the seedlings were transferred from light to darkness, it seemed likely that if this induction were effected at an earlier point in the life-history of the coleoptile it would be possible to record three peaks in the growth rate before elongation ceased. IX and X (Fig. 4) are replicate experiments in which the change to darkness was made at the 30th hour from soaking. Photographic recording was started at the same time, but owing to the shortness of the coleoptile at this stage many of the first photographs could not be measured accurately, so that values for these periods are not recorded. There is no clear indication of a peak about the 17th hour after the transfer to darkness, that is at about the 47th hour from soaking, but there is a peak 24 hours later than this, just after the 71st hour, with another just after the 95th hour. The change from light to darkness at the 30th hour apparently induced the 24-hour rhythm, but the manifestation of the first peak in the growth rate has been suppressed. The most probable explanation is that at about the time the first depression might be expected to occur the normal growth rate is increasing rapidly, and this acceleration partially counteracts the depression which otherwise would have been recorded about the 55th to the 65th hour. The tendency for the curves to flatten out during this period instead of rising suggests that the elimination of the depression is incomplete.

Expt. XI is similar to IX and X except that just before the 48th hour the plant chamber was removed from the apparatus and exposed to light for 5 minutes. (The light used was a 25-watt bulb under an orange shade at a mean distance of about 30 cm. from the coleoptiles.) Went (1941) has shown that exposure of *Avena* seedlings to light for a short time causes a light-growth reaction which is all over in about an hour. In Expt. XI there is some indication of a transient effect such as Went describes, but it is too small to be followed accurately in the photographs and it cannot be claimed as significant. While it is clear that the interruption of the dark period by a short exposure to weak light has no marked influence on the rhythm already induced at the 30th hour, the fact that the two peaks are about 1½ hours earlier than in the upper two graphs in Fig. 4 suggests that the light may have had some slight effect.

Before describing further experiments on interruptions of the dark period, mention may be made of Expt. XII (Fig. 4) which was designed to test whether the induction of the rhythm was in any way affected by the handling the seedlings received just before they were transferred to darkness. As in the preceding three experiments, the seedlings were removed from the dishes and fixed in position in the plant chamber just before the 30th hour, but instead of being placed immediately in darkness in the apparatus the plant chamber was exposed until the 48th hour to about the same intensity of red light as that to which the seedlings had previously been subjected. It was then placed in the apparatus and recording was started. The first peak in the growth rate occurred about 17 hours later, proving definitely that it is the change from light to darkness alone that induces the rhythm.

Interruption of dark period. Expts. XIII, XIV, and XV (Fig. 5) show the effects of interrupting the dark period by a much longer exposure to light than was used in Expt. XI. In XIII and XIV the seedlings were transferred from light to darkness at the 50th hour. The first peak occurred about 16·5 hours after this. Later the plant chamber was removed from the apparatus and exposed for 7 hours to red light of approximately the same intensity as that used during the early stages of germination. In XV the experiment was started earlier and the interpolated light period was extended to 12 hours. In each experiment the plant chamber was returned to the apparatus at the end of the light period and recording resumed. In all three experiments a new peak occurred 16–18 hours after the seedlings had been returned to darkness, but the time at which this peak occurs bears no fixed relation to the time of the earlier one. It is clear that the exposure to light for several hours abolished the previous rhythm and the subsequent transfer to darkness induced a new one. As shown by Expt. XI, 5 minutes' exposure to light of about the same intensity was insufficient to produce the changeover.

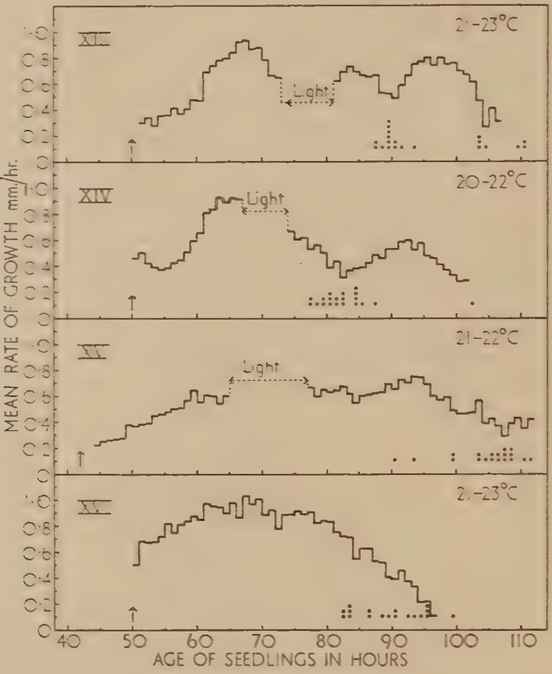

FIG. 5. In XIII and XIV the seedlings were transferred from red light to darkness at the 50th hour, and in XV at the 42nd hour. The dark treatment was interrupted by exposure to light for the periods indicated. The heights of the dotted lines show the mean growth rates during the light periods. As can be seen, peaks occur 16–18 hours after both the first and second transfer to darkness. XVI shows the mean growth rate of coleoptiles of seedlings kept throughout in red light. Recording started at the 50th hour.

Continuous red light. Expt. XVI shows the effects on the growth rate of retaining the seedlings in continuous red light. For this experiment certain modifications of the arrangements were necessary. The uppermost of the two light filters was removed and two 25-watt bulbs, each covered by a ruby glass shade, were installed in place of the 60-watt strip light. The red light was kept on continuously: consequently the usual practice of keeping the camera shutter permanently open could not be continued. Instead the shutter was made to open automatically for 1 minute every hour, and during the same period the lower strip light was switched on.

The result of keeping the seedlings continuously in red light is a typical grand period curve with a maximum at about 70 hours. There is no indication of any rhythmical variation in the rate of growth.

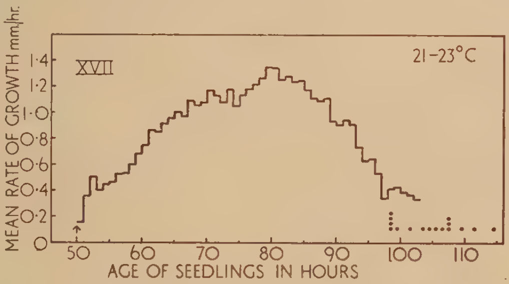

Fig. 6. Expt. XVII. Mean growth rate of *Triticum* coleoptiles germinated in red light and transferred to darkness at the 50th hour.

Continuous darkness. It was not feasible to use the photographic apparatus for automatically recording the growth rate of coleoptiles kept in constant darkness from the time of germination, owing to the excessive elongation of the mesocotyl which occurs under such conditions. Measurements were, however, made of the lengths of the coleoptiles in samples of 30 seedlings grown throughout in darkness and withdrawn at 10-hour intervals. The resulting curve of elongation agreed very closely with that of Schneider (1941) and is not recorded here. The results of the experiment gave no indication of a growth rhythm, although a final decision would need to be based on measurements made at much shorter intervals. However, since it appears unlikely that a rhythm would be induced in constant darkness, no further investigation of the matter was attempted.

Experiments on Triticum. Two experiments were carried out with *Triticum* (var. 'Holdfast'). The first (Expt. XVII) was a repetition of the methods used with *Avena* in Expts. I, II, and III, the seedlings being transferred from light to darkness at the 50th hour. The result is shown in Fig. 6. The rate of growth rises to a maximum at about the 80th hour and then declines. There is no rhythm.

In the second an attempt was made to record the elongation of the coleoptiles of seedlings grown in darkness in the photographic apparatus, since, unlike what happens in *Avena* when grown in darkness throughout, there is no elongation of the mesocotyl in *Triticum*. Unfortunately germination in the apparatus was very erratic. The results therefore are not recorded here, but they afforded no indication of the existence of a rhythm in the growth rate when the seedlings were germinated and grown in darkness.

The effects of temperature. The experiments so far described were carried out at temperatures within the range 20–25° C., but Fig. 7 shows the results of experiments on *Avena* carried out above and below this range. Expt. XVIII was within the range 20–28° C. and XIX approximately 10° lower, at 16–17° C. Both experiments were started when the seedlings were 50 hours old. At the lower temperature the mean growth rate was much smaller, the length of time during which elongation lasted was much longer, and the emergence of the leaves took place much later. The mean elongation from 50 to 95 hours at 20–28° C. was 33·24±1·45 mm., while at 16–17° it was only 19·62±0·31 mm. The periodicity, on the other hand, was unaffected, or, if a slight shortening occurred at the higher temperature, it was not more than 1 hour in 24, which is within the range of experimental error. Thus the much smaller variations in temperature which occurred in the main series of experiments would have been quite immaterial so far as the period of the rhythm is concerned.

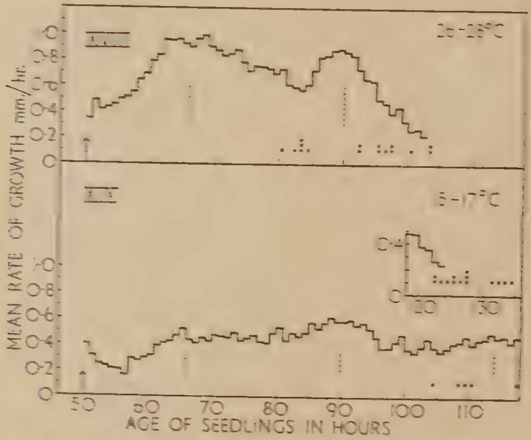

Fig. 7. Mean growth rate of *Avena* coleoptiles transferred from red light to darkness at the 50th hour. From then onwards the seedlings were at 20–28° C. in XVIII and at 16–17° C. in XIX. Vertical dotted lines are exactly 24 hours apart. Emergence of leaves shown by dots. In XIX the final part of the graph is shown as an inset.

At the low temperature the rhythm is not clearly marked, but there is no indication of a material change either in the times at which the peaks occur or in their distance apart; the period of the rhythm has certainly not increased. There is some suggestion of a third peak at 114 hours, 24 hours after the second.

These results are in contrast to those of Bünning (1931), who found that the periodicity of leaf movement in *Phaseolus multiflorus* was approximately 24 hours at 25° C., but increased to 30 hours at 15° C. and dropped to 19 hours at 35° C. On the other hand, Grossenbacher (1939), who studied the rhythmical changes in the amount of exudation from decapitated plants of *Helianthus annuus*, found that the amount of exudation decreased considerably when the temperature was lowered from 30° to 15° C., but the length of the cycle was only slightly, if at all, influenced by the change in temperature.

DISCUSSION

The occurrence in the *Avena* coleoptile of a growth rhythm of the kind described in this paper has not to our knowledge been reported previously, but certain other investigations are of interest in this connexion. Light-growth reactions in *Avena* have been studied by Vogt (1915), Sierp (1921), and Went (1941), but such reactions seem to fade away after 1 or 2 hours. Vogt (1915), Sierp (1918), and Schneider (1941) have compared the effects of prolonged light and darkness on elongation and have shown that light causes an increase in the growth rate, but this increase is followed by a diminution, so that the growth comes to an end sooner in light than in darkness and the total elongation is less.

Went and Thimann (1937, p. 59) give a graph of the growth rate from 0–100 hours based on measurements at 10-hour intervals of samples of 30–50 plants. The conditions under which the coleoptiles were grown are not stated, but Professor Went has kindly informed us in a personal communication that the plants were germinated under weak red light and were transplanted after approximately 24 hours and their lengths measured after different periods in darkness. This experiment therefore seems to be comparable to our experiments IX, X, and XI, where the seedlings were in red light for 30 hours and were then transferred to darkness. In Went's experiment and in ours there is a peak in the growth rate at about 70 hours, when the rate is about 1 mm./hr. The chief discrepancy is that in our experiments another peak occurred consistently some 24 hours later, for which there is no indication in Went's curve. This latter curve is, however, based on observations made only at intervals of 10 hours.

Kögl, Haagen-Smit, and van Hulssen (1936) found that the sensitivity to auxin of *Avena* coleoptiles of the same age varied according to the time of day, and similar variations have been recorded at Pasadena (Went and Thimann, 1937). Such variations must be ascribed to diurnal fluctuations in some external factor and cannot have any connexion with the 24-hour rhythm we have described. This rhythm shows no correlation with time of day, the time at which the maximum growth rate occurs being determined by the time at which the seedlings are transferred from light to darkness.

More recently Anker (1953) has reported a diurnal fluctuation in the rate of respiration of sections of 5-days old *Avena* coleoptiles which had been grown in darkness. His experiments lasted from 4 p.m. to 4 p.m. or later on the next day. From the time an experiment started, the rate of respiration gradually declined, the minimum rate being reached after about 12 hours. Then followed an increase in the oxygen uptake which reached a maximum about 4 p.m. on the second day and then declined once more. Anker states that the maximum rates of respiration were generally found in the early afternoon, but does not appear to have determined what would have happened if an experiment had been commenced at a different time of day. Until this is known the question whether or not there is any connexion between the diurnal fluctuation in the respiration rate of coleoptile sections and the endogenous rhythm in the growth rate of the intact coleoptile must remain open.

Rhythmical changes in exudation and guttation have been studied in several plants. In etiolated oat seedlings Engel and Friederichsen (1951) found a guttation pulse which reaches its maximum 3–6 hours after illumination. The position of the maximum is dependent on the instant at which the change from darkness to light occurs and can be brought about at any hour of day or night. In intermittent red light, fluctuations in guttation occurred with peaks about 12 hours apart although, with repetition, irregularity in the timing tended to increase.

Turning now to investigations on other plants, several instances have been recorded where a rhythm induced by 12:12-hour alternation of light and darkness persists after the plant has been transferred to a constant environment. Examples are leaf movement (Bünning, 1932), exudation (Grossenbacher, 1939), and growth of tissue cultures (Bünning and Enderle, 1950), (Enderle, 1951). Usually such rhythms seem to die away in the course of a few days, but, in the last example, cultures of carrot tissue which had remained in darkness for up to 4 months still showed variation in growth rate with a periodicity of approximately 24 (or 12) hours. The individual curves were no longer synchronous and the peaks did not occur at any particular time of day.

However, instances showing the greatest similarity to the growth rhythm in *Avena* are those in which an endogenous 24-hour rhythm appeared even though the plant had not been exposed to diurnal changes in environment. In such cases a stimulus of some kind seems necessary to start the rhythm. For example, Bünning (1931) has recorded a 24-hour rhythm in the leaf movements of *Phaseolus multiflorus* in darkness at constant temperature, the initiation of the rhythm being effected by 1 hour's exposure to red light, and Grossenbacher (1939) found that decapitated plants of *Helianthus annuus* grown under constant conditions showed 24-hour autonomic cycles of exudation, the first peak occurring about 12 hours after decapitation.

As Bünning (1932) has pointed out in the case of *Phaseolus multiflorus*, the plant must inherit the potentiality of carrying out diurnal, or approximately diurnal, movements although a diurnal rhythm of external conditions has not acted on it at any time during its individual life. Since he found that the kind of conditions which are operative during the ripening of the seeds do not affect in any way the characteristic features of the movements which appear later, he concluded that the potentiality for 24-hour autonomous movements was not acquired during the ripening of the seeds.

Assuming that the same holds good for *Avena*, one must conclude that the potentiality for a 24-hour growth rhythm is inherited, although the actual induction of the rhythm is dependent on a change from light to darkness. What is chiefly remarkable is the accuracy of the time-keeping, an accuracy which seems to be unaffected by moderate changes in temperature. Presumably the periodicity occurring under constant conditions is dependent on the rate of certain metabolic processes with an overall temperature coefficient close to unity. The elucidation of the basic reactions may be impracticable at present, but to determine the extent to which growth substances play a part in the final stages should be feasible. The actual rhythmical fluctuation in the growth rate may be due to variation in the concentration of such substances or to changes in the sensitivity of the tissues to their action.

An investigation of these points is now in progress.

We should like to express our thanks to Professor T. A. Bennet-Clark, F.R.S., for the interest he has taken in this investigation and for kindly providing the necessary facilities. The work was carried out with the aid of a grant from the Agricultural Research Council.

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NOTE

Studies on the Growth and Respiration of Roots

By L. J. Audus and A. Garrard

(Bedford College, University of London)

The authors wish to record their regrets and apologies for a misquotation in Vol. 4 on p. 333, line 5 from bottom. Contrary to what is stated, Coult (*Protoplasma*, **32**, 92, 1939) could not attribute the stimulation effect of agitation on root growth of *Sinapis* to increased aeration of the culture solution but thought a direct mechanical effect on the protoplasm to be more probable.

CORRIGENDA

'The Relation between Concentration of Hydrogen Ions and the Effect of Weak Acids or Bases on Metabolic Activity', by G. E. Briggs, Volume 5, No. 14

p. 265, equation 1. *For*

$$\frac{[P]+[A_i^-]+[OH_i^-]}{[A_0^-]\,[OH_0^-]}$$

read

$$\frac{[P]+[A_i^-]+[OH_i^-]}{[A_0^-]+[OH_0^-]}$$

p. 267, l. 3 from foot. *For* 1/47 *read* 1/57.